On the early management of hereditary hemochromatosis

Esther M.G. Jacobs
Properties: The melting point of iron is 1535°C, boiling point is 2750°C. Pure iron is chemically reactive and corrodes rapidly, especially in moist air or at elevated temperatures. Four allotropic forms, or ferrites, are known: α, β, γ, and δ, with transition points at 770, 928, and 1530°C. The α form is magnetic, but when iron is transformed into the β form, the magnetism disappears, although the lattice remains unchanged. Uses: Iron is vital to plant and animal life. In humans, it appears in the hemoglobin molecule. Iron metal is usually alloyed with other metals and carbon for commercial uses. (adapted from A.M. Helmenstine)

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On the early management of hereditary hemochromatosis

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Het is van wezenlijk belang om doelen en ambities te hebben – al zijn ze maar klein. Ze houden je jeugdig en hoopvol. *Paul Wilson*

Voor mijn ouders

Aan Arno, Mark en Paul
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CHAPTER 1

Background and outline of the thesis
HFE-gene related Hereditary Hemochromatosis (HH) is characterized by an autosomal recessive genetic mutation, which predisposes to an excess of iron absorption, leading to iron overload and its related atypical findings and clinical problems as elevation of liver enzymes, liver cirrhosis, hepatocellular carcinoma, diabetes mellitus, arthralgia and arthritis, cardiomyopathy and arrhythmia, impotence and loss of libido, fatigue, and anterior pituitary failure [1, 2]. In 1996, the genes leading to the HFE-related form of HH were for the first time described by Feder et al [3]. The prevalence of the most pathogenic HFE-mutation, the homozygous C282Y mutation, varies throughout the world. However, it occurs that approximately 9% of the North European population is heterozygous and 0.4% homozygous for the C282Y mutation [4].

Five stages have been proposed to be recognized in the development of HFE-related HH [5] (Figure 1). Still the pathogenesis of HFE-related iron overload is not yet fully elucidated. Mutations in the HFE-gene seem are associated with relative low hepcidin concentrations [6]. Hepcidin regulates the iron metabolism by disturbing the ferroportin release, which transports the iron from the duodenal villi cells and macrophages [7]. This leads to an elevated transferrin saturation and eventually to an elevated amount of non-transferrin-bound iron (NTBI) [7-9]. NTBI is thought to be the potential toxic form of iron responsible for tissue damage and the catalysis of redox reactions in the blood and endothelium as seen in hereditary hemochromatosis and in cardiovascular diseases [8, 10-14]. Once HH is diagnosed before irreversible organ damage has developed, the severeness of the disease and the number of deaths can be reduced easily by treatment of the iron condition using phlebotomies [15-18]. However, despite the high gene mutation frequency and the obviousness of the iron overload, the diagnosis of HH is often drawn only when irreversible organ damage has developed, as the early symptoms are relatively aspecific and often presenting at age of 40-50 years (Figure 1). So, screening for HH to prevent disease is desirable. There are several ways to identify for individuals with an elevated risk of HH, through e.g., i) clinical investigation of individuals with complaints pointing to HH, ii) population screening, or iii) screening of the families members of an individual clinically diagnosed with HH.

STUDIES INTO THE PATHOGENESIS AND EARLY MANAGEMENT OF HH

The aim of this thesis is to investigate a potential pathophysiologcal mechanism of NTBI in HH patients and to reveal an optimal early management strategy for HH. The disease entity of HH has undergone an evolution during the last decades. In 1889 iron overload was described for the first time in post mortem patients who died from ‘bronzed diabetes’. Later on it has been adapted to an autosomal recessive genetic disease with a high prevalence, but with varying, though treatable and
preventable, clinical features among genetic affected individuals. *CHAPTER 2* describes these changing aspects of HFE-related HH and its related dilemmas in early diagnosis of HH.

**FIGURE 1** Schematic view of the five stages of HFE-gene related HH in C282Y homozygous individuals during lifetime. Adapted from [5].

The raise in transferrin saturation is one of the earliest signs of HH, reflecting the disturbance in iron homeostasis (*FIGURE 1*). After transferrin becomes fully saturated, the excess of iron is unable to bind to transferrin and circulates as the potential toxic NTBI. The chemical structure of NTBI is heterogeneous and largely unknown, resulting in little consensus on its true level and how it should be measured. *CHAPTER 3* describes the results of the first international interlaboratory evaluation of NTBI from a common serum sample set, as a first step towards the standardization of NTBI quantification methods. In tandem with this laboratory research much work has been undertaken to clarify the pathophysiology of the molecular interactions of the various NTBI species with other molecules and cells, for instance the mechanism by which NTBI is involved in atherosclerosis and, HFE-gene related, cardiovascular diseases [11, 19, 20]. In *CHAPTER 4* the relationships between serum iron parameters, including NTBI, with plasma markers of inflammation and LDL oxidation, as part of the cholesterol metabolism, are investigated.

It is crucial that patients with HH are detected before irreversible organ damage has occurred. However, despite the high frequency of C282Y homozygosity in the Northern European countries, HH
is not often clinically diagnosed. Scepticism from physicians about the incidence of the condition is a serious barrier to early diagnosis through effective screening. This is also true for misunderstanding of the diagnostic criteria for HH, unfamiliarity with the multi-disease caused by iron overload and the belief that only those persons with skin bronzing, diabetes mellitus and hepatic cirrhosis have HH [2, 21-23]. This illustrates the importance of educating physicians on disease prevention strategy [24, 25], e.g. the implementation of a guideline for physicians to detect HH in an early, symptomatic stage [25]. In this respect \textit{CHAPTER 5} gives an impression of the impact of the implementation of a guideline for targeted detection of HH in an outpatient department of a university hospital.

Rather than targeted screening for clinical disease, population screening would offer the possibility of early detection of HH on a large scale, even before disease or irreversible organ damage has developed. Many reports have been written on the option of early screening on HH in the general population [26-37]. In fact, HH fulfils important criteria for mass screening programmes described by Wilson and Jungner four decades ago in 1968 [38]: There is a recognizable latent or early stage, there is a suitable test for examination, there are facilities for diagnosis and treatment, and there is an accepted effective treatment (\textit{TABLE 1}). However, the first, most important, item of these World Health Organization (WHO) [38] guidelines has remained unanswered: Is the disease for which screening is aimed an important health problem? Surprisingly and in contrast to most of the earlier studies, large and population controlled studies reported that a significant proportion of the C282Y homozygotes had no symptoms of disease at all [39-44]. Even more, studies which determined the degree at which homozygotes disappeared from the population as it ages, revealed no significant differences in the prevalence of untreated homozygotes among elderly populations compared to younger groups in several European countries [45-48]. These findings suggest that the penetrance of HH-related disease is by far not as high as was expected formerly. Indeed, the penetrance of the \textit{HFE}-gene mutations is probably influenced by other not yet distinguished factors, making population screening not a first choice method for screening for HH anymore.

As a third option of HH screening, family screening has been suggested, as method that lies in between screening of symptomatically suspect and screening of apparently healthy individuals. In family screening first-degree relatives of C282Y homozygous patients with clinically overt HH are screened for HH. After all, these family members are at relatively high risk of inheriting the same \textit{HFE}-gene mutations (risk for a sibling to be also C282Y homozygous is 25%) [49]. Moreover, the first-degree family members are most probably under the same genetic and environmental influences as is the diagnosed proband, which may determine also their phenotypic HH expression.
### TABLE 1
Principles for mass screening programs (World Health Organization, 1968) [38]

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<th>Principle</th>
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<tr>
<td>The condition sought should be an important health problem</td>
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<tr>
<td>There should be an accepted treatment for patients with recognized disease</td>
</tr>
<tr>
<td>Facilities for diagnosis and treatment should be available</td>
</tr>
<tr>
<td>There should be a recognizable latent or early symptomatic stage</td>
</tr>
<tr>
<td>There should be a suitable test or examination</td>
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<tr>
<td>The test should be acceptable to the population</td>
</tr>
<tr>
<td>The natural history of the condition, including development from latent to declared disease, should be adequately understood</td>
</tr>
<tr>
<td>There should be an agreed policy on whom to treat as patients</td>
</tr>
<tr>
<td>The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure in medical care as a whole</td>
</tr>
<tr>
<td>Case finding should be a continuing process and not a ‘once and for all’ project</td>
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In **CHAPTER 6** the rationale of family screening for hereditary hemochromatosis is explored. A database with anamnestic, biochemical and genetic data from probands with clinically proven C282Y homozygous HH, and their first-degree family members was built by performing the multicentre HEmochromatosis FAmily Study (HEFAS) study. First of all the morbidity and mortality in the first-degree family members of the HEFAS population is compared with the morbidity and mortality of an age and gender matched general population to determine whether there indeed is an important health problem among the first-degree family members of hemochromatosis patients. With this study, for the first time, the importance of the existing health problem within these hemochromatosis families can be demonstrated.

The design of HEFAS also made it possible to estimate the biochemical penetrance of the HFE gene mutations in the HEFAS families and to determine groups within these families at risk for iron overload condition. **CHAPTER 7** evaluates the levels of iron parameters among the first-degree HEFAS family members and identifies factors determining these levels, and as such attempts to predict who is at risk for iron accumulation within these families.

Understanding more about the expression of elevated iron parameters in the HEFAS families is only one stepping stone in a pathway from genetic predisposition to iron accumulation and the measured HH related disease among the first-degree HEFAS family members. This issue is addressed in
CHAPTER 8, which is aimed to quantify the relation between the genotype and the HH related sickness of the HEFAS population.

Finally, the results of the previous chapters are discussed in CHAPTER 9 and future perspectives on the realization of an effective strategy on early management of HFE-gen related HH are considered.
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CHAPTER 2

Changing aspects of HFE-related hereditary hemochromatosis and endeavours to early diagnosis

Submitted

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ABSTRACT

HFE-related hereditary hemochromatosis (HH) is an iron overload disease attributed to the highly prevalent homozygosity for the C282Y mutation in the HFE-gene. The pathophysiology of this error in the iron metabolism is not yet elucidated. Hepcidin is thought to be a crucial player nowadays. It is produced in the liver and regulates iron homeostasis by internalisation and subsequent degradation of ferroportin, thereby influencing iron transport out of the duodenal villi cells and macrophages. Iron overload is among others diagnosed by measurement of the serum iron parameters, i.e. serum transferrin saturation and serum ferritin, by a liver biopsy or by calculating the amount of mobilizable body iron withdrawn by phlebotomies. Clinical signs attributed to HFE-related HH include liver failure, arthralgia, chronic fatigue, diabetes mellitus and congestive heart failure. Organ failure can be prevented by phlebotomies starting before irreversible damage has occurred. Therefore, screening to facilitate early diagnosis is desirable in individuals at risk for developing HFE-related iron overload. In time it appeared that the clinical penetrance of the HFE-mutations was much lower than the 100% earlier assumed. This changed the opinion on a suitable screening facility from case detected screening, via population screening, to family screening as most appropriate method to restrict HFE-related disease. However, before implementation of family screening is a fact, for good evidence based screening policy, it is vital to have profound background information on the relevance of the specific health problem involved, on the clinical penetrance of C282Y homozygosity and on the effectiveness of the screening approach.

INTRODUCTION

Classical hereditary hemochromatosis (HH) is a disease related with iron overload with increasing physical complaints, organ failure, and poor survival. Therapy is relatively simple: Removing iron overload by phlebotomies, thereby preventing disease and increasing survival. After the discovery of its prime gene mutation, the C282Y mutation of the HFE-gene, large scale screening for HFE-related HH became feasible. However, along with the years it became clear that the traditionally low prevalence of patients with HH could not be fully ascribed to the ignorance of the medical workers, but mainly to the limited penetrance of the HFE-gene mutation. This review describes new insights in pathophysiology, diagnosis and penetrance of HFE-related HH, and its implications for secondary prevention and early treatment of the clinical disease.
HISTORY OF HFE-RELATED HEREDITARY HEMOCHROMATOSIS

One of the first to describe a clinical syndrome characterized by portal cirrhosis, diabetes mellitus and bronze skin pigmentation was Trousseau [1]. The name hemochromatosis was first used by von Recklinghausen (1889), describing post-mortem findings in patients who had died from ‘bronzed diabetes’ [2]. In 1935 Sheldon suggested a familial form of hemochromatosis [3], but it was not until 1975 that Simon et al described an autosomal recessive form of idiopathic hemochromatosis related to the HLA-A3 allele in the major histocompatibility complex (MHC) on chromosome 6. In 1996 Feder et al were able to isolate the HH gene in 85 percent of HH patients [4]. It was initially called HLA-H, as its organization and structure was similar to genes in the HLA region that coded for HLA-class I heavy chains. However, as a HLA-class I pseudo gene had already been named HLA-H, the newly identified hemochromatosis gene was renamed HFE (the abbreviation of HFE being surprisingly not otherwise specified) as proposed by the Genome Databank [5]. Until now, more than 30 allelic variants of the HFE-gene have been reported [6]. The most common mutation is C282Y that results from a transition at nucleotide 845 (845G → A), leading to substitution of tyrosine for cysteine. This alters the HFE protein and its association with β2-microglobuline, resulting in a decreased presentation of the HFE protein on the cell surface [7-9]. A second, though less important, HH-associated mutation occurs at nucleotide 187 of the HFE-gene, with a substitution of histidine for aspartate at nucleotide 63 (63H → D) [4]. Several other mutations of unknown significance are described.

PREVALENCE OF THE C282Y HFE-GENE MUTATION

The prevalence of the C282Y HFE-gene mutation varies throughout the world. The overall prevalence of homozygosity and heterozygosity for the C282Y mutation in European countries is 0.4% and 9.2%, respectively, with heterozygosity ranging form 1% in the Southern European countries to 24.8% in Ireland [10]. In North America an overall frequency of C282Y heterozygosity, regardless of the ethnical roots, was reported as 9.0%, whereas in the Indian subcontinent, and African, Middle Eastern and Australian populations prevalences of 0 to 0.5% percent were found [10]. For the Netherlands the percentages of C282Y homozygosity and heterozygosity are calculated at 0.2% and 12.0%, respectively [11].
PATHOPHYSIOLOGY

The evidence that the HFE protein is involved in regulating iron homeostasis was initially provided by the development of iron overload similar as in human HH in the \( \beta_2 \)-microglobulin knockout mouse, later confirmed in a mouse homozygous for the C282Y HFE-gene mutation and in human studies [12-17]. However, the exact role of the mutated HFE in the pathophysiology of iron overload remains to be elucidated. It has been suggested that the HFE protein modulates uptake of transferrin bound iron by undifferentiated intestinal crypt cells, thereby programming the absorptive capacity of enterocytes derived from these cells [18]. However, this so called crypt model remained controversial. Indeed, recently a normal iron metabolism was described despite the lack of HFE-gene expression in the duodenum [19]. In 2003, mice studies by Nicolas et al suggested that it is mainly the failure of hepcidin induction contributing to the pathogenesis of HH [20]. Hepcidin has been shown to regulate iron homeostasis by internalisation and subsequent degradation of ferroportin, a major cellular iron exporter protein in the duodenal villi cells and macrophages, which transport iron from the duodenal villi cells and macrophages to the plasma [21]. Consequently, the absence of hepcidin in mice leads to excessive iron release by enterocytes and macrophages, followed by circulatory and body iron overload [22]. This has even been demonstrated, before the detection of ferroportin, as HH monocytes and macrophages from HFE C282Y homozygotes released twice as much Fe(II) compared to cells from normal subjects [23]. Absent or very low hepcidin concentrations in man lead to a juvenile onset of the clinical iron overload disease, whereas moderate decreased hepcidin concentrations, in case of mutations in the HFE-gene, lead to relatively low and late onset of iron overload disease [24-27].

CLINICAL SIGNS AND SYMPTOMS IN HFE-RELATED HH

In 2000 an expert group described HFE-related HH as: “HH is an inherited disorder resulting from an inborn error of iron metabolism which leads to progressive loading of parenchymal cells in the liver, pancreas and heart. In its fully developed stage organ structure and function are impaired” [28]. Early clinical complaints encompass weakness, joint pain, palpitations, and abdominal pain, whereas massive iron overload will ultimately lead to arthritis, severe fatigue, chronic abdominal pain, liver enzyme elevations, liver cirrhosis, primary liver cancer, diabetes mellitus, hypopituitarism, hypogonadism, congestive heart failure, cardiac dysrhythmias, increased skin pigmentation and an increased risk of certain bacterial infections [28-35]. All symptoms are relatively unspecific, making it difficult to recognize them as related to iron overload. In addition the clinical penetrance of the HFE-
gene mutations is very variable [36-38]. First of all, this is due to gender and age of testing for iron overload. In men symptoms of the disease often become manifest at 40-50 years of age, in women mostly a decade later. Compared to women, men with HH have more mobilizable body iron [33]. Women are relatively spared by menstrual blood loss (15-25 mg) and pregnancies (750 mg each pregnancy) [33, 39, 40]. This does not mean, however, that (pre-) menopausal women cannot develop iron overload [41].

Next to age and gender, many other factors have been described to act in some degree upon the clinical penetrance of the HFE-gene mutations, including dietary iron intake, body mass index, pathological iron loss and the presence of toxins (e.g. alcohol) [42-44]. In addition, several other genes involved in the iron metabolism may act as HFE-genotype modifiers, although the results of the studies published are controversial. Described are e.g. hepcidin antimicrobial peptide (HAMP) mutations, transferrin receptor-2 missense variants, HJV mutations and haptoglobin variants are thought to amplify the amount of iron overload [45-48]. Although these multiple sequence variations are rare and still cannot explain most of the variation in the penetrance of the HFE-hemochromatosis, identification of the various iron overload determinants hopefully will improve our insights in the iron metabolism and help to predict which HFE-gene mutated patients are particular at risk for developing (early) iron overload and clinical complications.

**DIAGNOSIS OF IRON OVERLOAD**

Abnormal values for iron parameters in the serum, i.e. serum transferrin saturation (TS) and serum ferritin (SF) are strong indication for altered iron metabolism (Figure 1). In the literature different reference ranges are mentioned due to different populations examined. An elevated serum transferrin saturation above 45%, when doubting the value eventually measured a second time after fasting, in combination with an elevated SF level is highly suggestive for the presence of primary iron overload in the absence of other pathology like liver diseases and secondary causes of iron overload or alcohol abuse [41, 49-52]. The traditional gold standard for diagnosing iron overload is provided by a liver biopsy, although it is generally only required in presence of co-morbidities or strong suspicion of liver cirrhosis. A liver biopsy allows histochemical grading of iron (Perl's Prussian blue stain) or biochemical determination of hepatic iron concentration and calculation of the hepatic iron index (HII) (= ratio of hepatic iron concentration divided by age in years) [28, 49, 50]. A non-invasive tests which enables an appreciation of the amount of iron stored in the liver is the hepatic magnetic resonance imaging (MRI) [53-55]. The severity of iron overload can also be calculated from the number of phlebotomies required to deplete iron stores [28, 30]. Furthermore, HFE mutation analysis may help
to support the existence of iron overload [51, 56] and to detect individuals at risk for iron overload at younger age, when TS is often elevated, but SF still is normal (Figure 1).

<table>
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<th>Five stages of HFE-related HH [51]</th>
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<th>Composition of the population</th>
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<td>C282Y homozygosity with increased transferrin saturation, increased serum ferritin, and clinical symptoms manifesting organ damage predisposing to early mortality</td>
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<tr>
<td>C282Y homozygosity with increased transferrin saturation, increased serum ferritin, and clinical symptoms affecting the quality of life</td>
<td>III</td>
<td>III</td>
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<tr>
<td>C282Y homozygosity with both increased transferrin saturation, increased serum ferritin, but no clinical symptoms</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>C282Y homozygosity with increased transferrin saturation, but normal serum ferritin values and no clinical symptoms</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>C282Y homozygosity without biochemical or clinical symptoms (normal plasma transferrin saturation and serum ferritin)</td>
<td>0</td>
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**Figure 1** Schematic view of the five stages of HFE-related hereditary hemochromatosis (HH) together with the various strategies for screening on HH.

- Green, population screening strategy; yellow, family screening strategy; red, individuals who develop clinically important HH, targeted screening; horizontal red bar, individuals with C282Y homozygosity.

**TREATMENT OF HH**

The treatment of HH consists of venesection, as described by Davis [57]. It is safe, inexpensive and appeared to be effective, although the latter has never been proved. With the removal of 500 ml of blood, about 200 to 250 mg of iron is withdrawn from the body. Venesection is started when the SF levels are consistently above the upper limit of the reference range, pointing to body iron excess. Meanwhile, other causes for increased SF must be eliminated [41, 49-52]. Weekly phlebotomies are performed to withdraw excessive amount of iron, followed by yearly measurement of the serum.
ferritine and when necessary maintenance phlebotomies 1 to 8 times a year to maintain low body iron stores [28, 49, 58]. An alternative treatment for phlebotomy could be the less time consuming, though more expensive erythrocytapheresis, which extracts iron more quickly, as plasma and thrombocytes are not extracted from the circulation during the procedure. However, up to now it has been only utilized in small patient groups [59, 60]. A comparative study between the two treatment options is currently ongoing and will hopefully learn us more about the differences in (cost-) effectiveness of both treatments.

According to the experts’ opinion is that treatment should be continued until SF level is below the reference value [61]. Even so, diverse experts advise that next to venesection, dietary advices concerning iron overload management should be given, including moderation of alcohol intake and avoidance of iron, vitamin C supplements, uncooked seafood and drinking tea with meals which decreases iron absorption by formation of non-absorbable iron complexes [58, 62-66].

EVIDENCE OF PHLEBOTOMY BENEFITS

Before the introduction of phlebotomy treatment in 1935, survival of HH patients has been described as poor [30, 67]; the time from symptomatic presentation to death was 18 months [68]. However, many studies revealing a diminished survival among HH patients are based on clinical cohorts including mainly patients presenting themselves with late symptoms of severe iron overload [30, 67, 69, 70]. No controlled studies are available of phlebotomy treatment that allow a valid comparison of early versus delayed treatment. Niederau et al showed that iron depletion does attenuate weakness or lethargy, elevated liver enzymes and pigmentation. In contrast, arthralgia, impotence, electrocardiographic changes and diabetes mellitus remained in most cases, although the insulin dose could be reduced in 19 of the 46 insulin-dependent patients [30]. The HH participants included in McDonnell’s study also mentioned improvement in fatigue, skin bronzing, depression and abdominal pain [32]. Recently, Powell et al showed that phlebotomy treatment in 672 essentially asymptomatic C282Y homozygous subjects identified by either family screening or health checks, reduced the mean liver fibrosis score 7.5-fold [71]. In the best available evidence on the effects of phlebotomy treatment, Bomford and Niedereau reported that 7% to 23% of the patients clinically diagnosed with iron overload showed improvement of disease in the liver biopsies, 1% to 3% showed worsening [30]. Improvement in histological characteristics was more common in patients with less severe, precirrhotic liver disease (33% improved) compared to patients with cirrhosis (14% improved) [30]. Therefore, early diagnostic and therapeutic phlebotomy may reduce disease progress, especially when treatment is started before irreversible liver damage occurs [30, 71-73]. It has even been
suggested that timely started phlebotomy treatment is able to increase the survival to equal that of
the general population [30, 67, 69, 74].

FROM EARLY DIAGNOSIS AND TREATMENT TO DEATH PREVENTION

Despite the high frequency of the C282Y mutation and the obvious iron overload, the clinical
diagnosis of HH is often delayed until irreversible organ damage has developed, as early symptoms
are relatively non-specific. Even the more advanced complications are not always recognized as
symptoms of HH, unless specifically looked for. This is underlined by the findings of Powell et al [71]:
Through assessment of disease manifestation by clinical examination and liver biopsy in their
population of asymptomatic C282Y homozygous subjects, they found that hepatic iron overload was
already present in 56% of the males and 35% of the female subjects. Moreover, at least one, not yet
clinically diagnosed, HH related disease condition (arthropathy, diabetes mellitus, hepatomegaly,
hypogonadism or cardiac arrhythmia) was present in 30% of the males and 12% of the females [71].
This supports the statement that early screening for HFE-related HH is the key in early detection of
HFE-related iron overload, thereby preventing organ failure and death.

In an attempt to reappraise in general terms the evidence for screening and to determine what
attitude to screening should be adopted, in 1974 Whitby made a restatement of the principles of early
disease detection set up by Wilson and Jungner a few years earlier (TABLE 1) [75, 76]. Thereafter,
many reports have been written on the feasibility of early screening on HH in the general population
[77-86]. Indeed, HFE-related HH fulfils important criteria as described by Wilson & Jungner, and
Whitby: A recognizable latent or early stage, a suitable test for examination, facilities for diagnosis
and treatment and an accepted treatment [75, 76, 78, 87]. Still, one important item of these
guidelines remained unanswered: Is HH indeed an important health problem, not only for the
community, but also for the individual? [70, 75, 76]. Before the discovery of the HFE gene it was
assumed that every person homozygous for the, then unknown, C282Y mutation would eventually
accumulate sufficient iron to cause tissue damage and the resulting disease [41]. However, selection
bias, differences in case definition and population characteristics made it difficult to typify attributable
disease. This led to the troublesome fact that some authors found hemochromatosis related disease
in a high percentage of C282Y homozygous individuals, whereas others barely found any penetrance
of the HFE-gene mutations [29, 40, 71, 88-90]. Even more, some large and controlled studies
reported that a significant proportion of the C282Y homozygotes had no symptoms of disease at all,
questioning the importance of the involved health problem [37, 38, 91-94].
TABLE 1  Restatement by Whitby of the Wilson and Jungner principles for mass screening programs (World Health Organization, 1968) [75]

<table>
<thead>
<tr>
<th>Restatement of principles for mass screening programs</th>
</tr>
</thead>
<tbody>
<tr>
<td>The condition being sought should be an important health problem, for the individual and the community</td>
</tr>
<tr>
<td>There should be an acceptable form of treatment for patients with recognizable disease</td>
</tr>
<tr>
<td>The natural history of the condition, including its development from latent to declared disease, should be adequately understood</td>
</tr>
<tr>
<td>There should be a recognizable latent or early symptomatic stage</td>
</tr>
<tr>
<td>There should be a suitable screening test or examination for detecting the disease at the latent or early symptomatic stage, and this test should be acceptable to the population</td>
</tr>
<tr>
<td>The facilities required for diagnosis and treatment of patients revealed by the screening program should be available</td>
</tr>
<tr>
<td>There should be an agreed policy on whom to treat as patients</td>
</tr>
<tr>
<td>Treatment at the pre-symptomatic, borderline stage of a disease should favourably influence its course and prognosis</td>
</tr>
<tr>
<td>The cost of case-finding (which would include the cost of case finding and treatment) needs to be economically balanced in relation to possible expenditure on medical care as a whole</td>
</tr>
<tr>
<td>Case finding should be a continuing process and not a “once and for all” project</td>
</tr>
</tbody>
</table>

Next to settling the pre-screening prerequisite of the impact of the health problem of HFE-related HH, another principle of screening still not profoundly resolved, is the statement 8 added by Whitby (TABLE 1): Treatment at the presymptomatic, borderline stage of a disease should favourably influence its course and prognosis. In other words, early treatment should be more effective than started later in its developments and/or clinical phase.

The last screening issue to address is to decide which population is to be screened. In essence, searching for individuals with an elevated risk on HH can be performed at three population levels: i) clinical examination of individuals with complaints pointing to HH, i.e. targeted screening or case detection; ii) screening the families of a person clinically diagnosed with HH; and iii) population screening (FIGURE 1).

AD 1) CASE DETECTION

Medical examination of individuals with complaints pointing to HH would be a very direct way of detecting patients with HH. However, despite the high frequency of C282Y homozygosity in the Northern European countries HH was not often clinically diagnosed. This fed misunderstandings of physicians on the diagnostic criteria for HH, that used to encompass only the late stage combination of skin bronzing / hyperpigmentation, diabetes mellitus and hepatic cirrhosis, but should include more
disease entities. Furthermore, there is still unfamiliarity with the existence of the multiorgan disease and scepticism about the prevalence of the condition, all leading to a serious barrier to effectively screen for HH [52, 95, 96]. Therefore, it is important to educate physicians about HFE-related HH, e.g. the gene mutation frequency and its clinical penetrance, the diagnostic pathway and therapeutic options, when choosing this type of screening [97]. Thus, the implementation of a guideline for physicians on the targeted detection of HH in an early, symptomatic, stage could be beneficial [95]. Jacobs et al indeed introduced such a guideline and studied the impact of this introduction. They concluded that indeed the awareness for HH increased, though the implementation was troublesome at the cost of an increased rate of false positively new diagnosed HH patients, whereas 70% of the patients eligible for HH were still not tested [98]. Another drawback of this type of case detection is the risk that the newly discovered patients might have irreversible disease or organ failure at time of being diagnosed, diminishing the profits aimed for. Taken together, this screening strategy of case detection has its shortcomings for early disease detection.

**AD ii) FAMILY SCREENING**
In family screening first-degree relatives of C282Y homozygous patients with clinically detected HFE-related HH are screened for HH. After all, these family members are at relatively high risk of inheriting the same HFE-genotype (risk for sibling to be homozygous is around 25%) [36]. Moreover, they are likely to share genetic and environmental factors with the clinically diagnosed proband, which may also engrave their phenotypic expression of HH. From a theoretical point of view this screening strategy has potential, with an expected increased detection rate as well as higher effectiveness of early intervention [99-102].

**AD iii) POPULATION SCREENING**
In comparison to family screening, population screening offers the possibility of an even earlier and larger-scale detection of HFE-related HH. However, as mentioned above, although C282Y homozygosity is associated with health-threatening symptoms, these symptoms have been shown to occur in only a minority of the population derived C282Y homozygotes, making population screening not the first option of HH screening [70].
FUTURE INTERVENTION

HFE-related HH is a recognized clinical entity, with variable clinical penetrance. Screening and detecting those individuals at high risk of iron overload, before irreversible damage evolved, is likely to prevent organ detriment and death. From all the mentioned present screening options at this moment family screening is prone to be the most appropriate approach. However, before starting screening programs questions remain to be answered: Do C282Y homozygous individuals have a relevant health problem? What individuals are at risk to develop HFE-related iron overload and its accompanied disease? and; Is screening for these individuals cost-effective? To solve these questions we initiated the HEmochromatosis FAmily Study (HEFAS). From 224 probands homozygous for the C282Y mutation and presenting with clinically recognized symptoms of HH and 735 of their first-degree family members data have been collected regarding demographics, lifestyle (smoking, use of alcohol, diet), health, disease, family structure, including familial death rate, iron parameters and HFE-genotype. These data, that are currently analyzed, are likely to provide answers that are instrumental to prevent morbidity among yet unidentified individuals at risk for HFE-related HH.

In conclusion, the changing insights in the pathophysiological mechanisms of HFE-related iron overload, in the penetrance of the HFE-mutations and in the difficulties of early HH diagnosis, endeavour to investigate and develop more appropriate screening strategies to prevent iron overload related morbidity in those individuals at risk for iron accumulation.
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CHAPTER 3

Results of an international round robin for the quantification of serum non-transferrin-bound iron: need for defining standardization and a clinically relevant isoform

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ABSTRACT

Non-transferrin-bound iron (NTBI) appears in the circulation of patients with iron overload. Various methods to measure NTBI were comparatively assessed as part of an international interlaboratory study. Six laboratories participated in the study using methods based on iron mobilization and detection with iron chelators or on reactivity with bleomycin. Serum samples of 12 patients with hereditary (n=11) and secondary (n=1) hemochromatosis were measured in a three-day analysis, using four determinations per sample per day, making a total of 144 measurements per laboratory. Bland and Altman plots for repeated measurements are presented. The methods differed widely in mean serum NTBI (range 0.12-4.32 µmol/L), between sample variation (SD range 0.20-2.13 µmol/L and CV range 49.3-391.3%) and within sample variation (SD range 0.02-0.45 µmol/L and CV range 4.4-193.2%). The results obtained with methods based on chelators correlated significantly ($R^2$ range 0.86-0.99). On the other hand, NTBI values obtained by the various methods related differently with those of serum transferrin saturation (TS) when expressed both in terms of regression coefficients and NTBI levels at TS of 50%. Recent studies underscore the clinical relevance of NTBI in the management of iron-overloaded patients. However, before measurement of NTBI can be introduced into clinical practice, there is a need for more reproducible protocols as well as information on which method best represents the pathophysiological phenomenon and is most pertinent for diagnostic and therapeutic purposes.

INTRODUCTION

Iron is an essential metal in human metabolism. It is absorbed from the food in the intestine, entering the circulation bound to transferrin [1]. Under normal conditions 20-35% of the serum transferrin is saturated with iron. By sequestration on transferrin, iron is excluded from catalyzing reactions leading to the production of highly toxic reactive oxygen species and is safely transported through the blood [1]. However, when the transferrin becomes fully saturated, for example in hereditary and secondary hemochromatosis, excess iron is unable to bind transferrin and circulates as non-transferrin-bound iron (NTBI) [1-8]. NTBI may bind to putative ligands such as phosphates, citrate and albumin. Its exact biochemical nature, however, is unknown and it probably consists of a heterogeneous mixture of complexes whose composition might vary with the degree and type of iron-overload [2, 3, 9]. It is even known that some iron-overloaded patients with partially saturated transferrin also possess plasma NTBI [2, 10, 11]. This fraction of NTBI seems inaccessible to unsaturated transferrin and
apparently needs to be processed first by macrophages and liver and then discharged into the circulation as transferrin-accessible iron [2].

As iron binding to ligands such as phosphates, citrate and albumin is not as avid as its binding to transferrin, it is hypothesized that NTBI iron might be more readily available for catalyzing redox reactions in the blood and endothelium [9, 11, 12]. Additionally, various tissues might take up this loosely bound iron by a mechanism that is not as strongly regulated as the cellular uptake of transferrin bound iron, resulting in tissue iron accumulation and damage [13]. These mechanisms may contribute to pathologies seen in cardiovascular diseases and hereditary and secondary hemochromatosis [2, 4, 9, 12-15].

While few now doubt that NTBI exists, its nature is largely unknown, resulting in little consensus on its true level or how it should be measured. This may be the reason that research laboratories throughout the world measure NTBI using a variety of analytical approaches that differ in test principle and their exact practical application (i.e. choice of reagents and instruments).

The test principles of the methods described to quantify serum NTBI can roughly be divided into two groups. The first group of methods mobilizes NTBI by a shuttle molecule (e.g. EDTA or nitrilotriacetic acid (NTA)), followed by separation of the chelated iron from serum proteins using micro filters with a molecular weight cut-off of 30 000. The ultra filtrate can then be analyzed by atomic absorption, by spectroscopy, by high performance liquid chromatography (HPLC) and by inductive conductiometric plasma spectrometry (ICP) [16, 17]. Although these detection methods have a high reliability, they are very labour-intensive.

The second group of methods mobilizes and detects NTBI in the same reaction mixture, without separation of the serum proteins from chelated iron. One example is the bleomycin method which estimates the serum NTBI by measuring the redox active iron [18]. Ascorbic acid added to the serum reduces iron(III) to iron(II), which is then chelated by the anti-tumour antibiotic bleomycin. This complex binds to DNA and causes oxidative degradation of the deoxyribose moieties. These degradation products are measured by a colorimetric assay as their concentration is proportional to the iron present [18]. The problem with this assay is that it is an indirect measurement method that may be influenced by other substances in the serum with similar properties to iron [9]. Furthermore, not all iron(III) may be converted to iron(II) by ascorbic acid, and it is unclear whether all oligomeric iron will be detected in this assay [9]. Another example of the second group of NTBI measuring methods are the methods that use iron sensitive fluorescence probes, such as fluorescein labelled deferoxamine (Fl-DFO) or fluorescence labelled apotransferrin (Fl-aTf) to quantify NTBI in 96-well plates [19, 20]. These methods detect a diminished fluorescence signal when NTBI is present, compared to the signal given by normal serum. Originally DFO-coated wells were used, the DFO acting as a chelator to extract the NTBI from the sample, and a calcein-iron solution of which free
calcein induced the fluorescence signal. The free calcein originated from the calcein-iron complex of which the remaining available DFO extracted the iron when no more NTBI was available. Later, DFO-coated wells were replaced by normal wells and Fl-aTf solution was used to bind NTBI, thereby decreasing the fluorescence signal [19, 20]. A drawback of these methods is that they have the tendency to be affected by the local environment, such as serum colour or turbidity. This can be overcome by measurement of serum NTBI in absence and presence of an excess of chelator. The difference measured is the net NTBI related signal. The advantage of this method is its simplicity, making it suitable for routine screening of large numbers of serum samples.

An alternative way of ordering the NTBI detection methods is by separating them by means of the chemical characteristics of NTBI they measure. Redox activity of the serum iron is measured by the bleomycin method [18] and the method recently described by Esposito et al [21], whereas other methods measure the chelatable NTBI [9, 16, 17, 19, 20]. These latter chelating methods mutually differ in the choice of the chelator molecules for NTBI (i.e. NTA, oxalate), in reagents that block the unsaturated transferrin (i.e. Ga(III), Co(III), Mn(II)) and in final detection methods (i.e. ICP, HPLC, fluorescence).

There are diverse obstacles in the measurement of serum NTBI. First of all, the scavenging molecules used to bind the loose non-specific bound iron, can in principle also take iron from transferrin and ferritin [9, 16]. This can be minimized by using for example nitrilotriacetic acid (NTA), a scavenging molecule which is known to only mobilize a small fraction of transferrin and ferritin bound iron in the plasma [9, 16]. Secondly, scavengers can facilitate the transfer of NTBI to apotransferrin or monomeric forms of transferrin, falsely lowering the detectable load of NTBI, resulting in an underestimation or even negative NTBI results [2, 9, 17, 19]. This problem can be minimized by selectively blocking vacant transferrin sites, for example with Co(III) or less strong blockers as Ga(III) or Mn(III)), prior to the mobilization of iron [2, 16, 17, 20].

The NTBI-levels quantified by the diverse methods mentioned above might differ due to variation in assay principles, differences between laboratories in execution of the methods and by the mode and extent of the iron overload in the serum samples measured. All these methods currently continue to be used for various applications in the number of papers on NTBI without their results being mutually comparable. To investigate the relevance of the methods in the absence of a gold standard we compared i) the between sample variation of each method representing their suitability to measure NTBI in a wide variety of hemochromatosis samples and ii) the within sample variation of each method representing their reproducibility, iii) the repeatability of the methods with Bland and Altman plots and iv) the relation of each method to the transferrin saturation (TS). Therefore, we undertook a comparative study between the methods used routinely for research purposes throughout independent laboratories in Europe and Israel, using a common set of serum samples from
hemochromatosis patients. Here we report the results of the first international interlaboratory evaluation of NTBI from a common serum sample set. This may be the first step towards standardization of NTBI quantification methods.

MATERIAL & METHODS

STUDY DESIGN AND PARTICIPANTS
A prospective, repeated measurement design with 12 replications, for 12 different serum samples from iron overloaded patients, was used to assess concordance in quantitative NTBI analysis. Samples were analyzed in six laboratories, that routinely measured NTBI for research purposes, using eight NTBI determining methods, which consisted of five different chelating assays and one bleomycin assay (TABLE 1). The participating laboratories included laboratories in Israel (Jerusalem, n=1) and throughout Europe: Austria (Vienna, n=1), United Kingdom (London, n=1), Finland (Helsinki, n=1) and the Netherlands (n=2; Bilthoven and Nijmegen). They all received three identical vials with serum from each of the 12 patients. They were asked to quantify NTBI using their routine method(s) and to perform four measurements on each vial, on three different days, making a total of 12 measurements per patient sample. The participating laboratories were not informed on the biochemical characteristics of the samples. Samples were numbered in random order and shipped on dry ice to each of the participating centres. Laboratories were instructed to report their actual results.

SPECIMENS
Twelve serum samples were selected from the sample collection of iron overloaded patients on phlebotomy treatment in the Radboud University Nijmegen Medical Centre, the Netherlands. The patients of the selected samples were diagnosed with iron overload according to their serum TS and serum ferritin (SF) levels. Eleven of them were diagnosed as HFE-related hereditary hemochromatosis patients by the presence of either homozygosity for the C282Y mutation or compound heterozygosity for the C282Y / H63D mutations in the HFE gene. One patient was diagnosed with secondary hemochromatosis. Samples were selected as spanning a broad range of levels of serum iron parameters (serum TS, SF concentration). All patients were in different stages of their phlebotomy therapy.

Twelve ml of blood was drawn during phlebotomy therapy of each patient and collected without anticoagulant. Serum was separated after clotting by centrifugation of the blood at 2231g (within 60 min of sampling) and dispensed in polyethylene cryovials for storage at -40º C. Samples were defrosted and divided among cryovials, before freezing for shipment on dry ice at -70º C.
<table>
<thead>
<tr>
<th>Method</th>
<th>Lab*</th>
<th>Scavenger</th>
<th>Blocker</th>
<th>Detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>NTA</td>
<td>Co(III)</td>
<td>ICP-MS</td>
<td>[16]</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>NTA</td>
<td>Co(III)</td>
<td>HPLC</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>2</td>
<td>oxalate</td>
<td>Ga(III)</td>
<td>fluorescent-apo-transferrin</td>
<td>[20]</td>
</tr>
<tr>
<td>CHELATION</td>
<td>IIIB</td>
<td>3</td>
<td>oxalate</td>
<td>Ga(III)</td>
<td>fluorescent-apo-transferrin</td>
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<tr>
<td>IIIC</td>
<td>4</td>
<td>oxalate</td>
<td>Ga(III)</td>
<td>fluorescent-apo-transferrin</td>
<td>[20]</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>oxalate low</td>
<td>Mn(III)</td>
<td>calcein-Fe complex</td>
<td>[19]</td>
</tr>
<tr>
<td>V</td>
<td>5</td>
<td>oxalate high</td>
<td>Mn(III)</td>
<td>calcein-Fe complex</td>
<td>[19]</td>
</tr>
<tr>
<td>OTHER</td>
<td>VI</td>
<td>6</td>
<td>bleomycin, DNA</td>
<td></td>
<td>[18]</td>
</tr>
</tbody>
</table>

NTBI (non-transferrin-bound iron) is mobilized from its serum iron binding ligands by means of the scavengers listed. The blocker prevents binding of the released iron to unsaturated transferrin.

Lab, laboratory; NTA, nitrilotriacetic acid; ICP-MS, inductively coupled plasma mass spectroscopy; HPLC, high performance liquid chromatography; * The same numbers indicate the same laboratory.

**NTBI METHODS**

The methods used were divided into methods based on iron mobilization and detection with iron chelators (method I to V) and an assay to measure redox active iron (method VI, the bleomycin method) (TABLE 1). The first chelating method is named method I, using NTA to scavenge the serum NTBI, Co(III) to block any unsaturated transferrin sites available for the iron after NTA-scavenging and inductively coupled plasma mass spectrometry (ICP-MS) to detect the NTBI [16]. The method differed from the originally described method as follows. The samples were diluted 1:9, with NTA (8 mmol/L, pH 7.0) and Indium (10 mg/L), to obtain a sufficient amount of sample volume. Method II is similar to method I, except that samples were not diluted and that high performance liquid chromatography (HPLC) was used to detect the NTBI [16]. An ordinary HPLC system rather than a non-metal HPLC system was used for analysis. Method I and the second chelating method (method II) were carried out in the same laboratory. Methods IIIA, IIIB and IIIC were performed in three different laboratories. All three methods are based on the chelating assay described and published in 2001 by Breuer et al [20]. This assay uses oxalate as mobilizing agent, Ga(III) as blocker of vacant transferrin sites and fluorescein-apo-transferrin (Fl-aTf) to provide the fluorescence signal detectable.
with a fluorescence plate reader. Methods IV and V are also fluorescence methods based on the chelation of NTBI. These methods however, use Mn(II) as blocker of vacant transferrin sites and the iron chelator deferoxamine (DFO) to immobilize NTBI on the 96-well plate [19]. The fluorescence signal is provided by calcein, added to the assay as a calcein-iron complex. To mobilize the iron, oxalate is included in two different concentrations: a low concentration of 100 mmol/L (method IV) and a high concentration of 200 mmol/L (method V). Method VI estimates the serum NTBI by measuring the redox active iron and is termed “the bleomycin method” [18]. The method determinates NTBI by using a biological reagent, bleomycin, which forms a complex with redox active iron, causing a biochemical reaction that can be measured [18]. Standard curves were calculated for each series. Data were transformed to logarithmic values before calculation of the standard curve by linear regression in order to give weight to the standards with a low iron concentration (0.1-1 µmol/L) before fitting the curve.

SERUM IRON PARAMETERS MEASUREMENTS

Total serum iron was measured colorimetrically with use of ascorbate/FerroZine reagents (Hitachi 747, Roche). The interassay coefficients of variation or CVs were 4.3% and 5.9% for a low (15.7 µmol/L) and a high (46.0 µmol/L) control, respectively. The normal range was 10-25 µmol/L. Latent iron binding capacity (LIBC) was measured by adding a known quantity of Fe(III) to the serum sample, reducing it with ascorbate to Fe(II) and measuring it with FerroZine reagent as described above (Hitachi 747, Roche). The total iron binding capacity (TIBC) was calculated from Fe + LIBC = TIBC. The interassay CV was 2.0% and 4.3% for a low (36.7 µmol/L) and a high (72.0 µmol/L) control, respectively. Normal values ranged from 45-75 µmol/L. Serum TS was calculated as follows: (serum iron / TIBC) * 100% = serum TS (%). The SF was measured by a solid-phase, two-site chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, Cirrus). Normal ferritin values for pre- and postmenopausal women were 6-80 µg/L and 15-190 µg/L, respectively, and for men 15-280 µg/L.

STATISTICAL METHODS

The means of the four measurements (or three in case of an incidental outlier, see below for treatment outliers) were calculated, representing the NTBI outcome of one sample of one day. A linear mixed model was used to estimate the within sample SD and the between sample SD, for each method separately. The dependent variable was NTBI outcome and the independent random variable was sample (12 levels). The within sample SD and the between sample SD of each method are presented with the corresponding coefficient of variation (CV). Note that the SD reflects on average the absolute error and the CV reflects the relative error. The latter may be preferred in
cases in which the SD raises with increasing mean level, not unusual in laboratory measurements. A NTBI value was defined as an outlier within the four measurements of one sample on one day of a specific method, when its value was below or above three times the corresponding standard deviation (SD) from the mean. For this purpose the SD was calculated by using the resulting three measurements.

Pearson’s correlations between the individual sample mean values (of all 12 measurements per sample) obtained by the various methods were calculated. The Bland and Altman plots for repeated measurements are presented [22]. A linear regression model was used to study differences between the methods in their relationship between NTBI and TS. Again the dependent variable was the NTBI outcome. The independent covariable was TS and the independent class variable was method. As the relation between the SD (dependent) and the serum TS (independent) showed a correlation of 0.9, a linear scale for plotting the NTBI outcomes was chosen. The estimated regression coefficient of each method is presented with standard error (SE). Differences between regression coefficients were tested for statistical significance. Finally, the relation between the NTBI levels at a serum TS of 50% are presented, with differences in levels tested for statistical significance.

RESULTS

SERUM SAMPLE CHARACTERISTICS

Table 2 presents the serum sample characteristics. All samples are obtained from patients with iron overload disease, eleven patients with hereditary hemochromatosis and one patient with secondary hemochromatosis. The Hb varied from 6.9-9.7 mmol/L (normal range for men 8.1-10.7 mmol/L, for women 7.3-9.7 mmol/L), the serum iron concentration ranged from 5-45 µmol/L (normal values 10-25 µmol/L). The serum TS ranged from 7.4-97.0% (normal 10-45%), the SF concentration varied from 13- 2361 µg/l (normal values up to 280 µg/l). All the values are in agreement with the original iron overload disease and the different stages of treatment.

SERUM NTBI

A total of 955 NTBI measurements (of the scheduled 1152) were available for analysis (Table 3). The difference in numbers of results scheduled and actually presented are due to both missing samples and outliers. Missing values were obtained for method I for all the samples measured on the first day. For method II, one measurement of sample 5 was missing, next to the four measurements on the first day of sample 7. For method IIIC one measurement for all the three days was missing for
the samples 2, 5-12. Missed sample assignation can be ascribed to both deviations from the study design (missings in method I, IIIC and of sample five in method II) and failures (missings of sample seven in method II). TABLE 3 shows several missing values for the methods IV and V, due to execution difficulties with the prescribed protocol. All measurements of these methods were excluded for further analysis, as the obtained values turned out to be unreliably high, that is many exceeding the serum total iron concentration recorded in TABLE 2. Outliers were seen in the methods I (n=3, samples 3,4 and 8) and II (n=5, samples 1, 3, 5, 7 and 11) and these were also excluded from further analysis. Note that negative values of NTBI concentrations were found for the methods I, II, IIIB and IIIC. These values were accepted, as negative NTBI values are known to occur due to the combination of facilitated transport of NTBI by the scavengers to the vacant transferrin sites, which are not completely blocked by the used blockers Ga(III), Co(III), Mn(II), thereby falsely diminishing the measured amount of NTBI.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Disease of patients</th>
<th>Hb (mmol/L)*</th>
<th>Serum total iron (µmol/L)</th>
<th>Serum TS (%)</th>
<th>Serum ferritin (µg/L)</th>
</tr>
</thead>
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<td>5</td>
<td>7.4</td>
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<tr>
<td>2</td>
<td>comp</td>
<td>8.1</td>
<td>9</td>
<td>13.0</td>
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<tr>
<td>3</td>
<td>comp</td>
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<td>20</td>
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<td>24</td>
<td>59.0</td>
<td>887</td>
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<tr>
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<td>67.1</td>
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<td>10</td>
<td>homoz</td>
<td>9.1</td>
<td>45</td>
<td>85.5</td>
<td>50</td>
</tr>
<tr>
<td>11</td>
<td>sec</td>
<td>6.9</td>
<td>34</td>
<td>90.6</td>
<td>2361</td>
</tr>
<tr>
<td>12</td>
<td>homoz</td>
<td>9.4</td>
<td>36</td>
<td>97.0</td>
<td>1347</td>
</tr>
</tbody>
</table>

For presentation, the samples were ordered by increasing serum transferrin saturations.

homoz, HFE-gene related hereditary hemochromatosis with C282Y homozygosity; comp, HFE-gene related hereditary hemochromatosis with C282Y/H63D compound heterozygosity; sec, secondary hemochromatosis; TS, transferrin saturation.; * measured in whole blood.
TABLE 3 shows the mean and SD of the serum NTBI concentrations by sample and by method. The mean NTBI varied considerably between the methods under consideration i.e. methods I, II, IIIA-IIIC and VI. This was clearly demonstrated by the mean serum NTBI of sample 5 obtained by method IIIA, which was obviously higher than the outcome obtained by the related method IIIB; 4.26 µmol/L versus 0.43 µmol/L Fe, respectively. The bleomycin method (VI) generally showed remarkably lower values in mean NTBI concentrations compared to the other assays.

TABLE 4 presents the between and within sample SD and CV of each method. The within sample absolute (SD) and relative variations (CV) give an indication of the reproducibility of the individual assay and is based on repeated measurements of the same sample in a particular assay. This within sample variation varies considerably between assays. Methods IIIA and IIIIB reveal both a lower absolute and relative within sample reproducibility compared to the other methods, as their SDs and CVs were 0.19 µmol/L, 4.4% and 0.18 µmol/L, 29.5%, respectively. Especially the within sample SD's of method I and method II are relatively high compared to their mean NTBI levels (i.e. method I, CV 96.5% and method II, CV 193.2%).

The between sample variation gives an indication whether the method is suitable to measure a wide variation of NTBI levels in different hemochromatosis samples. This between sample SD and CV also varied greatly across the methods used. Method IIIA shows a relatively high between sample variance (SD 2.13 µmol/L) compared to the other chelating methods (SD 0.90-1.49 µmol/L) and especially to method VI (SD 0.20 µmol/L). Furthermore, the between sample SD of the methods is very high compared to the mean NTBI level measured in the sample population, except for method IIIA (method IIIA CV 49%, compared to CV of 108.6% to 391.3% for the other methods). Note that the method I, method II, method IIIB and method IIIC measured NTBI values below zero. This demonstrates that, although method IIIA seems to be preferable based on the between and within sample variation, further standardization of the chelating methods, is required to make a full comparison between these methods possible.

The Pearson correlation between the individual sample NTBI mean values obtained by the bleomycin method and that by the chelating methods was poor (p ns or p <0.05; range $R^2$ 0.51 to 0.64), whereas the mutual correlation between the NTBI sample means obtained by chelating methods was clearly significant (p <0.001, range $R^2$ 0.86 to 0.99).
## Table 3

Mean serum NTBI (SD) presented by sample and by method

<table>
<thead>
<tr>
<th>METHOD</th>
<th>Chelation</th>
<th>Redox activity</th>
<th>SAMPLES</th>
<th>ICP-MS</th>
<th>HPLC</th>
<th>Fluorescence (Fl-aTF)</th>
<th>Bleomycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
<td>IIIA</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-1.25</td>
<td>-1.35</td>
<td>1.22</td>
<td>3.41</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.05</td>
<td>-1.33</td>
<td>0.51</td>
<td>5.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.21</td>
<td>-0.22</td>
<td>2.80</td>
<td>9.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19</td>
<td>-0.15</td>
<td>3.24</td>
<td>18.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.04</td>
<td>-0.02</td>
<td>4.26</td>
<td>27.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.38</td>
<td>0.31</td>
<td>4.10</td>
<td>24.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.73</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.66</td>
<td>0.28</td>
<td>5.22</td>
<td>38.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.59</td>
<td>1.72</td>
<td>7.02</td>
<td>70.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.10</td>
<td>0.64</td>
<td>6.76</td>
<td>56.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.97</td>
<td>1.03</td>
<td>6.09</td>
<td>65.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.31</td>
<td>1.08</td>
<td>6.56</td>
<td>79.37</td>
</tr>
</tbody>
</table>

The mean and SD are expressed in µmol/L Fe; Sample, blood sample from a patient as indicated in table 2; n, number of measurements (when not indicated n = 12, i.e. four measurements at three different days); ICP-MS, inductively coupled plasma mass spectroscopy; HPLC, high performance liquid chromatography; Fl-aTF, fluorescent-apo-transferrin; Calcein-Fe, calcein-iron complex.
**Figure 1** shows the difference in mean serum NTBI (µg/L) between two methods against the average of the mean values of these two methods. Method VI, i.e. the bleomycin method, was not evaluated for this relation as i) the NTBI means of method VI remained close to zero for the samples 1-8 and ii) the Pearson correlation between the sample NTBI mean values obtained by this bleomycin method and that by the chelating methods was poor. **Figure 1** shows clearly nonuniform differences throughout the range of the measurements.

Consequently, the limits of agreement are too far apart suggesting in some plots a (very) poor agreement. In such cases log transformation or regression techniques may help to provide better (nonuniform) limits. However, in our case it is more obvious to study the methods in relation to the serum TS of each sample (representing the true quantity) as described in the next section.

**Table 4** Between sample and within sample variation in NTBI concentration, by method

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean SD</th>
<th>Between samples SD CV</th>
<th>Within samples SD CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (ICP)</td>
<td>0.59 0.90</td>
<td>152.5 96.5</td>
<td></td>
</tr>
<tr>
<td>II (HPLC)</td>
<td>0.23 0.90</td>
<td>391.3 193.2</td>
<td></td>
</tr>
<tr>
<td>CHELATION</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIA (FL-aTF)</td>
<td>4.32 2.13</td>
<td>49.3 4.4</td>
<td></td>
</tr>
<tr>
<td>IIIB (FL-aTF)</td>
<td>0.61 1.30</td>
<td>213.1 29.5</td>
<td></td>
</tr>
<tr>
<td>IIIC (FL-aTF)</td>
<td>1.37 1.49</td>
<td>108.6 24.7</td>
<td></td>
</tr>
<tr>
<td>OTHER</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI (Bleomycin)</td>
<td>0.12 0.20</td>
<td>166.7 15.3</td>
<td></td>
</tr>
</tbody>
</table>

The mean is calculated as the mean outcome of the means of each of the three days (each day four measurements) found for each method on one patient sample. SD and CV are calculated using a linear mixed model. Mean and SD are presented in µmol/L; CV is presented in %; between sample SD and CV reflect the variation in the population; within sample SD and CV reflect the variation between the replicates of one method.

**Serum TS versus NTBI**

**Figure 2** displays the relation of the serum NTBI with the serum TS for the methods I to IIIC. For the same reasons as mentioned in the previous section method IV was not evaluated for this relation. NTBI levels rise linearly with TS in all assays shown. **Table 5** shows the regression coefficients of this relation between the increase in serum NTBI (µmol/L) and the rise in serum TS of the different methods, calculated as increase in NTBI per unit increase in TS. This regression coefficient increases from method I (and II) to method IIIB (and IIIC) being highest for method IIIA (i.e.
FIGURE 1 The difference in mean serum NTBI (µg/L) between two methods against the average of the mean values of these two methods. The solid lines represent the mean difference with 95% limits of agreement of an individual sample. The limits of agreement are obtained by using the within-sample standard deviation of the methods as required in case of repeated measurements.
regression coefficient of 0.028 µmol/L/% (0.029 µmol/L/%) to 0.048 µmol/L/% (0.042 µmol/L/%) and 0.068 µmol/L/%, respectively). A similar pattern was observed for the mean level of NTBI at serum TS of 50%, except that here the mean level of method I (0.35 µmol/L) and IIIB (0.26 µmol/L) did not differ significantly.

Figure 2  The mean serum NTBI (non-transferrin-bound iron) against serum TS (transferrin saturation) of a sample, by method.  
The lines indicate the estimated mean NTBI using a multivariate linear regression model, the symbols indicate the observed mean NTBI.  
Method I: , thick solid line, R = 0.82; method II: x, thick long dashed line, R = 0.85; method IIIA: , thick short dashed line, R = 0.96; method IIIB: , thin solid line, R = 0.96; method IIIC: , thin long dashed line, R = 0.94.
### Table 5

<table>
<thead>
<tr>
<th>Method</th>
<th>Regression coefficient (SE)</th>
<th>NTBI level at TS 50% (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II (HPLC)</td>
<td>0.029 (0.003)</td>
<td>0.01 (0.09)</td>
</tr>
<tr>
<td>I (ICP)</td>
<td>0.028 (0.003)</td>
<td>0.35 (0.09)</td>
</tr>
<tr>
<td>CHELATION</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIIB (FL-aTF)</td>
<td>0.048 (0.003)</td>
<td>0.26 (0.09)</td>
</tr>
<tr>
<td>IIIC (FL-aTF)</td>
<td>0.042 (0.003)</td>
<td>0.98 (0.09)</td>
</tr>
<tr>
<td>IIIA (FL-aTF)</td>
<td>0.068 (0.003)</td>
<td>3.76 (0.09)</td>
</tr>
</tbody>
</table>

Different numbers in the same column indicate statistical significant difference, p <0.05, using a multivariate linear regression model.

SE. standard error; NTBI, non-transferrin bound iron; TS transferrin saturation; NTBI level at TS is presented in µmol/L. The regression coefficient indicates the amount of increase in NTBI (in µmol/L) with an increase of 1 unit (1%) of serum TS.

### DISCUSSION

The present round robin comparatively assessed serum NTBI concentrations measured with different NTBI quantification methods, 5 different chelating methods and a bleomycin assay. The study showed a considerable intra- and inter-method variation for the quantification of serum NTBI levels of hemochromatosis patients in different stages of their phlebotomy treatment. Moreover, serum NTBI values obtained by the various methods correlated differently with those of TS. Despite these differences between methods, the serum NTBI concentrations obtained with the methods based on chelators correlated significantly.

Correlations of NTBI values were most significant between NTBI values found by the methods that belonged to the same method group, as was illustrated by the strong mutual correlations between the NTBI means measured by the chelating methods. In contrast, correlations between the chelating methods and the bleomycin method were either weak or non-existent. As the bleomycin method was originally developed to measure NTBI levels in serum samples with a TS of 80% or higher, it could very well be that the correlation between the chelating methods and the bleomycin method increases when more samples with iron saturations above this level are included.

Although the methods significantly correlated, they widely differed in mean NTBI as well as in the between sample variation and the within sample variation. NTBI results by methods with a high between sample variation, such as method IIIA (SD 2.13 µmol/L) showed a wider variation of levels...
for the different hemochromatosis samples in comparison with methods with a low between sample variation such as the bleomycin method (SD 0.20 µmol/L). The low SD for the bleomycin method suggests that this method is less suitable to measure NTBI in a variety of samples of hemochromatosis patients. However, the absence of a gold standard assay and an international calibrator precludes the definite choice of the most optimal method based on these between sample variations. Methods with a relatively low within sample variation, such as method VI (SD 0.02 µmol/L) and IIIA (SD 0.19 µmol/L) and IIIB (SD 0.18 µmol/L) gave more reproducible NTBI outcomes than methods with more elevated within sample variations such as I (SD 0.57 µmol/L) and II (SD 0.45 µmol/L). The combination of both a relatively high between and low within sample variation, as for the methods IIIA (SD between 2.13 µmol/L; SD within 0.19 µmol/L) and IIIB (SD between 1.30 µmol/L; SD within 0.18 µmol/L) suggest that these methods have the most optimal profile for reproducible measurements of a wide range of NTBI levels of hemochromatosis patients. In this respect, the between and within sample SD profile of methods I and II suggest a less favourable assay. However, the negative values of the methods I, II, IIIB and IIIC make a full comparison difficult.

The differences between the methods I and II and the other chelating methods IIIB and IIIC were also reflected in differences between the regression coefficients considering the relationship of the measured NTBI levels with serum TS, i.e. with increasing TS, the NTBI levels obtained by the methods I and II raise less than those obtained by methods IIIB and IIIC. The observed absolute and relative variation in serum NTBI results of the diverse methods might have several causes. First of all, the use of different chelating methods with each their own specific scavengers and blockers could have influenced the results, as these various scavengers and blockers each have their own chemical behaviour [2, 16]. A second explanation for the variation in NTBI outcomes could be that all laboratories differently interpreted the original method description and made various minor adjustments, which might have influenced their results. For example, chelating methods IIIA, IIIB and IIIC that originated from the same source [20] used identical blockers and scavengers, but differed in the use of equipment (i.e. quality of glassware, plastics and the fluorescent plates), reagents (i.e. the preparation and final concentration of the Ga(III) solution, use of iron free reagents, fluorescence labelling procedures) and the construction of the calibration curves (i.e. ratio of Fe to NTA, Fe concentration range). It is outside the scope of the present study to exactly identify the main cause(s) of the differences between these 3 methods. Also, some published methods are difficult to reproduce. This is illustrated by the unreliably high NTBI concentrations obtained by the methods IV and V. In a multistep assay of this type, instability of calcein complexes or iron contamination might have played a role [23].
Since the NTBI assay needs to detect very low concentration of iron, contamination can be a serious problem and a third source of variation between labs. Iron can be present in the whole laboratory environment, reagents materials and equipment. It appeared to us that is was very difficult for laboratories to exclude contaminant iron to prevent erroneous outcomes. The effect of iron contamination can be illustrated by the measurements performed on sample seven with method II on day one, using only routinely cleaned tubes, leading to four NTBI results with a mean of 3.85 µmol/L. This in contrast to the quadruplicate measurements on the same sample on the following 2 days, when especially iron-free prepared tubes were used, revealing mean NTBI outcomes of only 0.93 µmol/L and 1.28 µmol/L, respectively. A fourth explanation for the observed differences in mean NTBI levels between methods might be the existence of various NTBI isoforms. The various methods may select for different forms of NTBI; in other words, isoforms may not be equally well detected by each of the NTBI assays. This might particularly explain the differences in NTBI values between the chelating methods and the bleomycin method. Furthermore, isoforms might vary with the degree and type of iron overload [9]. So, the results of the present NTBI method comparison study on samples of patients with hereditary hemochromatosis may not simply be extrapolated to samples of patients with other diseases such as β-thalassemia, or patients on chemotherapy. This dependency of the measured NTBI concentrations on both mode and extent of disease and the method used, might explain the results of the following three recent studies: i) Esposito et al, using serum samples of patients with β-thalassemia instead of hereditary hemochromatosis, did find a correlation between the serum NTBI concentration measured by a chelating method and a new and alternative method for measuring the serum redox active iron (Labile Plasma Iron) [21]; ii) Sahlsted et al, using serum from haematological stem cell transplant patients, found lower serum NTBI concentrations when NTBI was measured by the bleomycin method, than when the amount of NTBI was calculated from the shift of apotransferrin into the monoferric form of transferrin after a single apotransferrin infusion [24] and iii) von Bonsdorff et al, using serum from patients with haematological malignancies, found higher NTBI-values by their chelating method than by their bleomycin method [18]. The latter results were confirmed by the present study with samples from a different patient group.

From the differences between the methods described above we conclude that standardization of a research method such as NTBI is urgently needed before it continues to be used in diverse fields of research or even in clinical practice. We propagate that all papers on NTBI-methods should include thorough characterization of the methodology, analytical control and construction of standard curves. Furthermore the linearity for an indicated dynamic range, within and between day variability, and information on how iron contamination was avoided, should be described.

In conclusion, the present study shows that NTBI values differ considerably between methods. These differences are largely caused by i) variations in the procedures, ii) iron contamination and iii)
differences in the nature of the NTBI complexes or isoforms measured by the methods. Although recent studies underscore the clinical relevance of NTBI [2, 10, 13, 14, 25], it remains unclear which method best represents the pathophysiological phenomenon and is most pertinent for diagnostic and therapeutic purposes. Furthermore, our study clearly shows that laboratory protocols should be more thoroughly standardized, to decrease the variation of NTBI results within and between laboratories. Therefore, we conclude that before NTBI can be introduced into clinical practice additional information is needed on its nature and relevance and more robust quantification-methods should be developed.

ACKNOWLEDGEMENTS

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Non-transferrin-bound iron is associated with plasma level of soluble intercellular adhesion molecule-1 but not with \textit{in vivo} low-density lipoprotein oxidation.

\textit{Atherosclerosis}, 2006, \textit{epub}

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ABSTRACT

Background: Excess body iron is associated with increased cardiovascular disease risk, possibly via non-transferrin-bound iron (NTBI)-mediated enhancement of inflammation and oxidation of low-density lipoprotein (LDL). Methods: We assessed this proposed atherosclerotic mechanism of body iron by determining the relationship of levels of serum iron parameters, including NTBI, with plasma markers of inflammation and LDL oxidation in 232 subjects who visited the outpatient clinic for hemochromatosis family screening. Results: Plasma level of soluble intercellular adhesion molecule-1 (sICAM-1) was positively related to serum ferritin (SF) (standardized beta coefficient 0.16) and to NTBI (0.185) and negatively to total iron-binding capacity (TIBC, -0.166). Significant higher levels of sICAM-1 were found for subjects in the highest quartile of NTBI compared to the lowest quartile of NTBI (122 µg/L (107-141) and 106 µg/L (89-125), median (interquartile range), p<0.001). Odds ratio of subjects having sICAM-1 level above 134 µg/L (75th percentile) in the highest and lowest quartile of NTBI amounted 2.3. White blood cell count was positively related to SF (0.149). High sensitivity C-reactive protein, interleukin 6, interleukin 8, oxidized LDL, oxidized LDL/apolipoprotein B and IgG and IgM antibodies to oxidized LDL were not related to any of the markers of iron status. Conclusion: Excess body iron, reflected by elevated SF and NTBI and decreased TIBC, is associated with increased plasma level of sICAM-1 but not with markers of in vivo LDL oxidation.

INTRODUCTION

Although not confirmed by a meta-analysis of prospective studies [1], epidemiological and experimental data suggest evidence for a potential role of iron in atherosclerosis [2]. It is hypothesized that free iron-catalyzed oxidation of low-density lipoprotein (LDL) is causally involved [3, 4]. In healthy adult men, intracellularly most iron is contained in haemoglobin, myoglobin and SF, and in plasma the glycoprotein transferrin efficiently binds iron. Thus, levels of free iron are reduced to nearly zero. When body iron rises to levels above normal, however, part of it may not be safely stored. Iron then binds to ligands with less affinity than transferrin, such as citrate, phosphates, and albumin, and may be available for redox reactions [5]. Moreover, also in the presence of unsaturated transferrin, non-transferrin-bound iron (NTBI) can be detected in serum [6, 7]. NTBI can catalyze the production of reactive hydroxyl radicals out of the relatively innocuous phagocyte- and endothelium-derived superoxide radicals [8]. Hydroxyl radicals can damage DNA, lipids and proteins. Oxidized LDL are pro-inflammatory, they attract monocytes via induction of chemokines, and upregulate scavenger receptor expression on macrophages. The scavenger receptors subsequently recognize
NTBI is associated with plasma level of sICAM1 but not with *in vivo* LDL oxidation

Oxidized LDL and mediate their uptake into the cells. This process leads to lipid accumulation in the vessel wall and fatty streak formation, the beginning of atherosclerosis. This proposed atherosclerotic mechanism of iron can be verified by measuring serum levels of NTBI in relation to markers of LDL oxidation and markers of inflammation. Assays to measure NTBI and oxidized LDL are available [9-11], and we have recently compared different methods to measure NTBI [12]. Although the comparability of the methods appeared to be rather low and (inter)national standardization is still lacking, one of the methods has an optimal profile for reproducible measurements of a wide range of NTBI. Therefore, this method is suitable to relate NTBI with other variables within one population. Up to now, in no study oxidized LDL and NTBI were measured in one population. Others did previously find that in healthy subjects who participated in feeding studies, NTBI was not associated with measures of *in vitro* LDL oxidation (lag-time, rate of oxidation, and total dienes formed) [13]. However, this is a non-physiological assessment of LDL oxidation. Furthermore, in a few studies oxidized LDL has been studied in relation to other variables of iron status in populations having normal or sub-normal iron status [14-17]. These studies, however, did not yield concordant results, probably because the stored body iron marker used (SF) is an inappropriate marker of free (redox-active) iron [18].

In this study we explore the relation of variables of iron status, including NTBI, with serum markers of inflammation and of LDL oxidation, including oxidized LDL, in a population of subjects expected to cover a wide range of serum iron status.

**MATERIALS & METHODS**

**SUBJECTS**

Study subjects were patients diagnosed with hereditary hemochromatosis and family members (n=173 from 69 families) and spouses (n=59) who visited the outpatient clinic for hemochromatosis family screening at the Radboud University Nijmegen Medical Centre between February 2002 and September 2004. Excluded were subjects with cardiovascular disease (CVD), diabetes mellitus or infectious diseases (C-reactive protein > 20 mg/L), and subjects receiving statin therapy or iron chelation medication, or using antioxidants. Pregnancy and alcohol abuse were also exclusion criteria. A participant was considered to have CVD when he had been diagnosed with angina pectoris, myocardial infarction, arrhythmia, stroke, or peripheral arterial disease, or when he had undergone intervention procedures (balloon angioplasty, coronary bypass surgery or aortic aneurysm surgery)

Of each participant a self-reported medical history, including current drug use and family history of
premature vascular disease (onset <60 years in first degree family), and information on alcohol consumption, physical activity, smoking behaviour and blood donorship, were attained by questionnaire and reviewed by a research assistant. The waist circumference was measured at the level of the umbilicus, the hip circumference was measured at the level of the trochanter major, and the waist-to-hip ratio was calculated. Height and weight were measured and body mass index (kg/m²) was calculated. Blood pressure was measured using an automated monitor (Welch Allyn Medical Products, model 5200-103A, Skaneateles Falls, NY, USA) in supine position and blood was drawn by venipuncture. Iron status (except NTBI) and plasma lipids were determined in fresh samples. For determination of NTBI, apolipoprotein B (apoB), and markers of inflammation and LDL oxidation, aliquots of serum and EDTA-plasma (with and without saccharose (0.6% w/v) as cryopreservant) were stored at –80 °C. The protocol was approved by the hospital ethics-committee and written informed consent was obtained from all participants.

**BIOCHEMICAL MEASUREMENTS**

**IRON STATUS**

Serum total iron and serum total iron binding capacity (TIBC) were determined by routine clinical chemistry methods on the Hitachi 747 (Roche) and SF was measured by a solid-phase two-site chemiluminescent immunometric assay using the Immulite 2000 (Diagnostic Products, Cirrus); serum TS was calculated as a percentage of serum total iron divided by TIBC. NTBI was measured in serum by a method using iron-sensitive fluorescence-labelled apotransferrin, as described previously [9] and recently evaluated by our group in an interlaboratory comparative study [12]. The assay has an optimal profile for reproducible measurements of a wide range of NTBI levels (range 0-7 µmol/L, within-samples CV = 4.4%).

**LIPIDS AND APOLIPOPROTEIN B**

Plasma cholesterol and triglycerides were determined using commercially available reagents on the Hitachi 747 analyser (Boehringer Mannheim, Germany). High-density lipoprotein (HDL)-cholesterol was determined with the phosphotungstate/Mg²⁺ method [19]. LDL-cholesterol was calculated with the Friedewald formula. ApoB was quantified by immunonephelometry [20].

**MARKERS OF INFLAMMATION AND LDL OXIDATION**

High-sensitivity C-reactive protein (hsCRP) was measured by enzyme-immunoassay according to the instructions from the manufacturer (Dako, Glastrup, Denmark). Coefficient of variation was less than 6%.

Concentration of soluble intercellular adhesion molecule-1 (sICAM-1) in plasma was measured with a
NTBI is associated with plasma level of sICAM1 but not with in vivo LDL oxidation.

Interleukin-6 (IL-6) and interleukin-8 (IL-8) were determined with Pelipair® reagent sets purchased from Sanquin (Amsterdam, The Netherlands). White blood cell count was determined automatically using the Advia 120 haematology analyser (Bayer, Tarrytown, USA).

For measurement of oxidized LDL we used commercial non-competitive ELISA (Mercodia, Uppsala, Sweden). The assay uses monoclonal antibody 4E6 to specifically capture oxidized apoB from the sample, which is subsequently detected with an antibody to apoB. Intra- and interassay coefficients of variation of the assay amounted 6% and 7%, respectively. As shown previously by our group [22] and confirmed recently by Holvoet et al [23], duration of storage at –80ºC did not influence the amount of oxidized LDL measured, provided that the EDTA samples had been collected carefully and had not been thawed.

Circulating antibodies (IgG and IgM) to oxidized LDL were measured as described previously [24]. In short, samples were incubated in wells of microtiter plates precoated with native or oxidized LDL, and bound antibodies were detected using peroxidase-conjugated antibodies from goat specific for human IgG or IgM (Sigma-Aldrich, Steinheim, Germany). Results are expressed as the mean optical density values at 450 nm from duplicate measurements, and the antibody titer to oxidized LDL is calculated by subtracting the binding to native LDL from the binding to oxidized LDL. With this subtraction method our assay not only corrects for specific binding to native LDL, but also for non-specific binding of each sample to the wells of the microtiter plate. The same batch of native and oxidized LDL was used for all determinations of antibodies to oxidized LDL in this study.

**HFE GENOTYPING**

The HFE C282Y genotype was determined by an automated method using minor-groove-binding DNA oligonucleotides (MGB probes) as described previously [25]. The presence of a C282Y allele was confirmed by conventional PCR with restriction fragment length polymorphism analysis.

**STATISTICAL ANALYSIS**

Levels of SF, TS, hsCRP, sICAM-1, IL-6, IL-8 and IgG antibodies to oxidized LDL showed skewed distribution and statistical analyses were based on transformed (natural logarithm) data. ANOVA was used to test for differences between sexes and between subgroups of smoking, alcohol consumption, physical activity and blood donation. Correlations between numeric variables were assessed with Spearman’s correlation. Multiple linear regression analysis was used to analyse the relation between markers of iron status and markers of inflammation and LDL oxidation. Age, gender, systolic blood pressure, waist/hip, plasma LDL cholesterol, smoking status, and alternately serum iron, TIBC, TS,
SF and NTBI were entered into the model as explanatory variables. Markers of inflammation and LDL oxidation were used as outcome variables. No adjustments for multiple comparisons were made, following the arguments of Rothman [26] and Perneger [27, 28], which suggest that a Bonferroni adjustment is overconservative in a study with many non-independent variables. To take into account the concern of multiple comparisons, a P-value of 0.025 was taken as the level for statistical significance. All of the analyses were performed using SPSS 12.0.1 for Windows.

RESULTS

The study population consisted of 232 subjects with a mean age of 46 year, almost equal distribution of sexes, and relatively normal body mass index, blood pressure and plasma lipids (TABLE 1). About equal numbers of subjects smoked, had quitted smoking, or had never smoked. A quarter of the study population consumed no alcohol and one third did not sport regularly (less than two hours/week). Twenty percent of the study subjects donated blood regularly (500 ml twice a year as a donor, letting blood by the patients excluded). Iron status of the study population is presented in TABLE 2. Levels of serum iron ranged from 4 to 56 µmol/L and were higher in males than in females; 24% of subjects had levels above 25 µmol/L, the upper border for normal values. TIBC varied from 35 to 89 µmol/L and was similar in males and females; 13.9% of subjects had levels lower than 45 µmol/L and 3.4% of subjects had levels higher than 75 µmol/L (lower and upper border for normal values, respectively). TS ranged from 4.8 to 102.2% and was higher in males than in females; 6% of subjects had TS above 80%. SF levels ranged from 2 to 3010 µg/L; 23% of the males had levels above 280 µg/L and 21.8% of the females had levels above 190 µg/L (upper borders for normal values of males and females, respectively). NTBI levels varied from 0.44 to 6.21 µmol/L.

Within the whole population, as expected, NTBI correlated positively with serum iron (r=0.81, p<0.001), TS (r=0.84, p<0.001) and SF (r=0.28, p=0.001), and negatively with TIBC (r=-0.37, p<0.001). These correlations were similar for males and females.

Markers of inflammation and LDL oxidation were not different between sexes (TABLE 2). HsCRP correlated positively with oxidized LDL (r=0.21, p=0.001), sICAM-1 (r=0.26, p<0.001) and white blood cell count (r=0.26, p<0.001), and sICAM-1 correlated with IL-8 (r=0.27, p<0.001). Other correlations between variables of inflammation and LDL oxidation were weaker (r<0.20) or not significant.

Seventy-one subjects (29 males and 42 females) did not carry the C282Y mutation in the HFE gene, 111 were heterozygous (53 males and 58 females) and 50 subjects (28 males and 22 females) were homozygous for the mutation. Of the subjects homozygous for the mutation, 78% underwent regular bloodletting to lower their body iron. Still, the homozygous subjects had significantly higher levels of
NTBI is associated with plasma level of sICAM1 but not with in vivo LDL oxidation.

serum iron, TS and NTBI, and lower levels of TIBC compared to subjects not carrying the mutation (TABLE 3). Levels of markers of iron status of subjects heterozygous for the C282Y mutation in the HFE gene were intermediate between those of homozygotes and non-carriers. Similar differences between genotypes were found within males and females separately, although significance was not always reached due to lower numbers (not shown). No differences were observed in markers of inflammation and LDL oxidation between the different genotypes of C282Y of the HFE gene (not shown).

TABLE 1 Characteristics of the study population (n=232)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>46 (14)</td>
</tr>
<tr>
<td>Gender (m/f)*</td>
<td>47/53</td>
</tr>
<tr>
<td>Physical examination</td>
<td></td>
</tr>
<tr>
<td>Body mass index (kg/m²)*</td>
<td>25.0 (22.9-27.7)</td>
</tr>
<tr>
<td>Waist/hip</td>
<td>0.86 (0.08)</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>81 (11)</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>133 (19)</td>
</tr>
<tr>
<td>Plasma lipids and apolipoprotein B</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.3 (1.0)</td>
</tr>
<tr>
<td>Total triglycerides (mmol/L)*</td>
<td>1.29 (0.96-1.86)</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.32 (0.37)</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>3.3 (0.9)</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/L)</td>
<td>962 (233)</td>
</tr>
<tr>
<td>Life style characteristics</td>
<td></td>
</tr>
<tr>
<td>Smoker (present/past/never)*</td>
<td>28/35/37</td>
</tr>
<tr>
<td>Alcohol consumer (yes/no)*</td>
<td>76/24</td>
</tr>
<tr>
<td>Sporting regularly (yes/no)*</td>
<td>65/35</td>
</tr>
<tr>
<td>Blood donor (yes/no)*</td>
<td>20/80</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD), unless indicated otherwise. HDL, high-density lipoprotein; LDL, low-density lipoprotein; * presented as median (interquartile range); * presented as percentage.
## Chapter 4

### Table 2: Plasma markers of iron status, inflammation and LDL oxidation of the study population (n=232)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Males (n=110)</th>
<th>Females (n=122)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Iron status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum iron (µmol/L)</td>
<td>22.9 (9.4)</td>
<td>19.8 (7.4)†</td>
</tr>
<tr>
<td>Serum TIBC (µmol/L)</td>
<td>54.3 (8.8)</td>
<td>56.5 (9.8)</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>37.8 (28.7-53.7)</td>
<td>33.3 (24.9-42.4)†</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>125 (50-280)</td>
<td>70 (38-173)†</td>
</tr>
<tr>
<td>Serum NTBI (µmol/L)</td>
<td>2.96 (1.25)</td>
<td>2.47 (1.07)†</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HsCRP (mg/L)</td>
<td>1.1 (0.4-2.5)</td>
<td>1.5 (0.7-3.6)</td>
</tr>
<tr>
<td>sICAM-1 (µg/L)</td>
<td>113 (100-132)</td>
<td>115 (94-135)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.2 (0.5-2.3)</td>
<td>1.0 (0.5-2.0)</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>6.0 (3.2-9.2)</td>
<td>4.8 (3.0-4.8)</td>
</tr>
<tr>
<td>White blood cells (10⁹/L)</td>
<td>6.4 (2.0)</td>
<td>6.7 (1.8)</td>
</tr>
<tr>
<td><strong>LDL oxidation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidized LDL (U/L)</td>
<td>58 (21)</td>
<td>55 (17)</td>
</tr>
<tr>
<td>Oxidized LDL/apoB (U/mg)</td>
<td>0.059 (0.011)</td>
<td>0.058 (0.010)</td>
</tr>
<tr>
<td>IgG antibodies to oxidized LDL (OD₄₅₀)</td>
<td>0.36 (0.24-0.49)</td>
<td>0.34 (0.23-0.52)</td>
</tr>
<tr>
<td>IgM antibodies to oxidized LDL (OD₄₅₀)</td>
<td>0.54 (0.34)</td>
<td>0.62 (0.37)</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD), unless indicated otherwise. TIBC, total iron-binding capacity; NTBI, non-transferrin-bound iron; hsCRP, high-sensitivity C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1; IL-6, interleukin-6; IL-8, interleukin-8; LDL, low-density lipoprotein; apoB, apolipoprotein B; IgG, immunoglobulin G; IgM, immunoglobulin M; OD, optical density.

* presented as median (interquartile range); † p < 0.025 compared to males (ANOVA).

Next, we analysed the contribution of iron status to plasma levels of markers of inflammation and LDL oxidation. Age, gender, waist/hip ratio, systolic blood pressure, smoking status and plasma LDL cholesterol (not for oxidized LDL/apoB ratio) were included in the model as possible confounders (as established by bivariate correlation analyses (Spearman) and ANOVA). After adjustment, hsCRP, IL-6, IL-8, oxidized LDL, oxidized LDL/apoB and IgG and IgM antibodies to oxidized LDL were not related to any of the markers of iron status (Table 4). Plasma level of sICAM-1 was positively related to SF (standardized beta coefficient 0.16) and to serum NTBI (0.185) and negatively to serum TIBC.
NTBI is associated with plasma level of sICAM1 but not with in vivo LDL oxidation.

White blood cell count was positively related to SF (0.149). When the population was divided into quartiles of NTBI, significant higher levels of sICAM-1 were found for subjects in the highest quartile of NTBI compared to the lowest quartile of NTBI (122 µg/L (107-141) and 106 µg/L (89-125), median (interquartile range), p<0.001). Odds ratio of subjects having sICAM-1 level above 134 µg/L (75th percentile) in the highest and lowest quartile of NTBI amounted 2.3 (0.98-5.6, 95% confidence interval).

**TABLE 3** Iron status of the study population stratified by the C282Y genotype of the HFE-gene

<table>
<thead>
<tr>
<th></th>
<th>C282Y non-carriers (n=71)</th>
<th>C282Y heterozygotes (n=111)</th>
<th>C282Y homozygotes (n=50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum iron (µmol/L)</td>
<td>18.4 (4.8)</td>
<td>21.4 (8.4)*</td>
<td>25.2 (11.0)*</td>
</tr>
<tr>
<td>Serum TIBC (µmol/L)</td>
<td>59.4 (8.9)</td>
<td>55.7 (9.0)*</td>
<td>49.4 (7.7)*†</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>30.4 (24.7-36.1)</td>
<td>37.1 (27.4-46.6)*</td>
<td>47.7 (32.7-77.7)*†</td>
</tr>
<tr>
<td>Serum ferritin (µg/L)</td>
<td>94 (44-174)</td>
<td>88 (50-231)</td>
<td>54 (25-199)</td>
</tr>
<tr>
<td>Serum NTBI (µmol/L)</td>
<td>2.25 (0.75)</td>
<td>2.78 (1.19)*</td>
<td>3.18 (1.42)*</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD), unless indicated otherwise. TIBC, total iron binding capacity; NTBI, non-transferrin-bound iron. * presented as median (interquartile range); * p < 0.025 compared to non-carriers; † p < 0.025 compared to heterozygotes (t-test).

**DISCUSSION**

The present study comprises the first in vivo data supporting an association between NTBI and plasma level of sICAM-1. We observed a significant positive correlation of serum NTBI with the plasma level of sICAM-1, but not with variables of in vivo LDL oxidation. Recently, Kartikasari et al showed that NTBI from human sera upregulates the expression of adhesion molecules on human umbilical vein endothelial cells (HUVEC) in vitro and promotes the extent of monocyte adhesion [29]. The increase of adhesion molecule expression was shown to be due to increased intracellular oxygen-derived free radical formation, resulting from an augmentation of the level of metabolically and catalytically reactive cytoplasmic labile iron [29, 30].

The presently observed association of NTBI with plasma level of sICAM-1 could contribute to the suggested increased risk on CVD at excess body iron. Endothelial ICAM-1 plays a critical role in the monocyte recruitment to the extravascular compartment by mediating adhesion and transmigration of the cells to the vascular endothelial wall [31]. In addition, adhesion molecules have an important role...
TABLE 4  Linear regression analysis of the association of markers of inflammation and LDL oxidation with markers of iron status

<table>
<thead>
<tr>
<th></th>
<th>Iron</th>
<th>TIBC</th>
<th>TS</th>
<th>SF</th>
<th>NTBI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HsCRP</td>
<td>-0.069 (p=0.29)</td>
<td>0.021 (p=0.75)</td>
<td>-0.090 (p=0.17)</td>
<td>0.056 (p=0.39)</td>
<td>-0.045 (p=0.50)</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.025 (p=0.71)</td>
<td>-0.166 (p=0.02)</td>
<td>0.064 (p=0.34)</td>
<td>0.16 (p=0.02)</td>
<td>0.185 (p=0.007)</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.150 (p=0.27)</td>
<td>-0.040 (p=0.57)</td>
<td>-0.124 (p=0.07)</td>
<td>-0.077 (p=0.25)</td>
<td>-0.086 (p=0.21)</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.073 (p=0.28)</td>
<td>0.038 (p=0.58)</td>
<td>0.045 (p=0.50)</td>
<td>0.083 (p=0.21)</td>
<td>0.100 (p=0.14)</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>-0.101 (p=0.12)</td>
<td>0.012 (p=0.86)</td>
<td>-0.088 (p=0.17)</td>
<td>0.149 (p=0.02)</td>
<td>-0.114 (p=0.08)</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>0.046 (p=0.33)</td>
<td>0.088 (p=0.08)</td>
<td>0.025 (p=0.60)</td>
<td>0.06 (p=0.21)</td>
<td>0.007 (p=0.88)</td>
</tr>
<tr>
<td>Oxidized LDL/apoB</td>
<td>0.117 (p=0.08)</td>
<td>0.03 (p=0.63)</td>
<td>0.137 (p=0.04)</td>
<td>0.054 (p=0.42)</td>
<td>0.036 (p=0.59)</td>
</tr>
<tr>
<td>IgG antibodies to</td>
<td>-0.001 (p=0.99)</td>
<td>0.029 (p=0.69)</td>
<td>0.023 (p=0.74)</td>
<td>-0.061 (p=0.38)</td>
<td>-0.073 (p=0.30)</td>
</tr>
<tr>
<td>oxidized LDL</td>
<td>-0.023 (p=0.73)</td>
<td>-0.081 (p=0.24)</td>
<td>-0.032 (p=0.63)</td>
<td>-0.001 (p=0.99)</td>
<td>-0.011 (p=0.87)</td>
</tr>
</tbody>
</table>

Statistics based on linear regression; given are standardized beta coefficients (p-value) adjusted for age, gender, waist/hip ratio, systolic blood pressure, smoking status and plasma LDL cholesterol.

# not adjusted for LDL cholesterol; TIBC, total iron binding capacity; TS, transferrin saturation; SF, serum ferritin; NTBI, non-transferrin-bound iron; hsCRP, high-sensitivity C-reactive protein; sICAM-1, soluble intercellular adhesion molecule-1; IL-6, interleukin 6; IL-8, interleukin 8; LDL, low-density lipoprotein; apoB, apolipoprotein B.

in the initiation and progression of atherosclerotic disease [32, 33], probably through alterations in gene expression that lead, for example, to secretion of cytokines and metalloproteases. Previously, Ridker et al found a significant association between increasing concentration of sICAM-1 and risk of future myocardial infarction in apparently healthy men [34]. More recently, Kondo et al, in a longitudinal study, observed that sICAM-1 was associated with carotid atherosclerosis progression, independently of traditional risk factors and CRP, in outpatients treated for traditional risk factors for CVD [35]. These findings suggest that levels of sICAM-1 may have potential value to predict
NTBI is associated with plasma level of sICAM1 but not with *in vivo* LDL oxidation.

![Regression plots of sICAM-1 with SF, TIBC and NTBI.](image)

**FIGURE 1** Regression plots of sICAM-1 with SF, TIBC and NTBI. Linear regression analysis of plasma soluble ICAM-1 levels with SF (A), TIBC (B) and NTBI (C). sICAM-1, soluble intercellular adhesion molecule-1; SF, serum ferritin; TIBC, total iron-binding capacity; NTBI, non-transferrin-bound iron.

atherosclerosis progression.

Up to now serum NTBI and plasma markers of inflammation and *in vivo* LDL oxidation were not studied before in one population. A few studies addressing the inflammation/oxidation hypothesis of iron-overload, used SF as variable reflecting iron status [14-17]. Weak but significant relationships were observed for SF with plasma levels of oxidized LDL, normalized for serum LDL cholesterol concentration, in the male subgroup of healthy Caucasians [14], with the cholesterol autoxidation...
products 7β-hydroxycholesterol and 7-ketocholesterol in eastern Finnish men [16], and with C-reactive protein in young women but not in young men [36]. On the other hand, no association was found between blood donation-induced low SF levels and plasma levels of adhesion molecules and oxidized LDL [17], and among the participants of the Atherosclerosis Risk in Communities (ARIC) study, no association existed for SF with auto antibodies to malondialdehyde-modified LDL [15]. However, SF is an inappropriate marker of free (redox-active) iron [18]. In contrast to NTBI, ferritin is hypothesized to be not available for catalyzing free radical formation. In addition, serum NTBI and SF correlate only weakly. Furthermore, SF is an acute phase reactant that is elevated by infection and may be elevated by inflammation. For these reasons, associations of markers of inflammation and in vivo LDL oxidation with NTBI differ from those with SF, and we feel that measurement of NTBI itself should be preferred when assessing a possible association between iron status and inflammation and oxidation. Moreover, in the studies mentioned above, iron status of the participants was normal or even subnormal. We assume that, if a relationship exists between iron status and measures of inflammation or LDL oxidation, this will be most apparent when subjects with elevated serum iron status are included.

In the present study, we used a population of subjects covering a wide range of serum iron status, and we found a clear association of serum NTBI with sICAM-1 and weaker, but also significant, associations of SF and total iron binding capacity with sICAM-1. These data are in favour of a direct in vivo oxidative stress-promoting effect of NTBI. The oxidative stress, however, does not reach a level high enough to promote oxidative modification of apolipoprotein B, since we did not find an association of circulating oxidized LDL with NTBI. Previously, we measured copper-induced LDL oxidizability of two groups (23 subjects each) of hereditary hemochromatosis heterozygotes (heterozygous for the C282Y mutation and wild-type for the H63D mutation in the hemochromatosis gene) showing clear differences in serum NTBI [37]. We found no difference in lag-time between the groups with high and low serum NTBI. Only the rate of in vitro LDL oxidation and the amount of dienes formed were slightly but significantly decreased at elevated serum NTBI. We hypothesized that at increased level, NTBI may cause a continuous low-grade oxidative pressure in such a way that molecules most prone to lipid peroxidation are constantly oxidized, and due to this decrease in oxidizable substrates, the rate and extent of in vitro oxidation is reduced. In line with this, others found that levels of F2-isoprostanes, one of the most specific measures of lipid peroxidation available, esterified in plasma lipoproteins, were increased soon after rapid infusion of iron in excess of transferrin binding capacity [38]. Consistent with lag-time, we found no difference in the vitamin E content of LDL between the groups with high and low serum NTBI [37], probably because vitamin E is successfully regenerated by antioxidants abundantly present in plasma. Thus, although oxidized LDL particles would be rapidly cleared from the circulation by the reticulo-endothelial system, our
previous and present findings of unaltered vitamin E and oxidized LDL levels at elevated NTBI, suggest that it is improbable that elevated NTBI enhances formation of oxidized LDL. Therefore, our findings do not support the hypothesis that increased body iron status is associated with increased oxidative modification of LDL. Still, the early lipidperoxidation products that do get formed, may contribute to atherogenesis directly. For instance, oxidized phospholipids have been demonstrated to mediate many atherogenic processes, from monocyte migration into the vessel wall to thrombus formation.

In conclusion, elevated iron status is associated with increased plasma level of sICAM-1 but not with measures of in vivo LDL oxidation. Enhanced recruitment of inflammatory cells to the extravascular compartment by increased endothelial expression of ICAM-1 may contribute to the suggested increased risk on CVD at excess body iron.

ACKNOWLEDGMENTS

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Impact of the introduction of a guideline on the targeted detection of hereditary hemochromatosis

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ABSTRACT

Background: In 1998 a clinical guideline for the targeted and accurate and early detection and treatment of HFE-related hereditary hemochromatosis (HH), that comprises a test for the causative HFE-gene mutations, was introduced at the outpatient department.

Methods: The impact of this guideline was evaluated retrospectively. Data were acquired from medical records of patients with discharge diagnosis codes suggestive for HH (n=878 patients), obtained from a period before (n=422) and after guideline introduction (n=456).

Results: Combined measurements of serum transferrin saturation and serum ferritin rose from 12.2% (n=53) to 29.5% (n=138, p<0.001), leaving 70% of the patients eligible for HH not tested for iron parameters. The HFE-gene mutation detection test was correctly used in 11 (40.7%) of 27 tested patients and improperly interpreted in 6 (22.2%) of these 27 patients. Five new HH patients were diagnosed before and 13 after introduction. Seven of these 13 patients appeared to be incorrectly diagnosed, due to misinterpretation of laboratory results.

Diagnostic costs of case detection for each accurately diagnosed patient were 2,380 Euro before and 2,600 Euro after introduction of the guideline.

Conclusion: Evaluation of the introduction of a practical guideline for targeted HH detection, reveals a low compliance with the guideline, resulting in both a small percentage of patients tested for HH and overdiagnosis of HH. Therefore, the introduction of the guideline should be combined with a more appropriate implementation strategy, which includes education on its most critical points, i.e. the indication and interpretation of the iron parameters and the HFE-genotype.

INTRODUCTION

The medical and scientific interest in HFE-related Hereditary Hemochromatosis (HH), iron overload disease, quickly expanded after the discovery of the causative C282Y and H63D mutations in the hemochromatosis (HFE-) gene in 1996 [1]. The C282Y-mutation is now the most common autosomal recessive mutation in people of northern European descent, with an estimated prevalence of the genetic susceptibility for HH by homozygous C282Y mutation, of 1 in 200-250 persons [2, 3]. Complaints that can be attributed to iron overload are fatigue, arthralgia and cardiac rhythm disorders [4-6]. Furthermore, diabetes mellitus, elevated liver enzymes, liver cirrhosis, hepatocellular carcinoma and cardiac failure can be considered as signs of HH [5, 7, 8]. The latter three being the most common cause of death in untreated HH patients [6, 7].

The first step in the diagnosis of HH consists of the recognition that these symptoms and signs in
combination with persistent elevated serum transferrin saturations (TS) and elevated serum ferritin (SF) concentrations may be attributed to HH, especially when these laboratory values remain unexplained [4, 5, 9, 10]. The diagnosis HH is confirmed by the presence of homozygosity for the C282Y mutation, by compound heterozygosity for the C282Y and H63D mutation in the HFE-gene and by iron overload shown in a liver biopsy, on exclusion of secondary causes of iron tissue accumulation such as ineffective erythropoiesis, haemolysis, concomitant liver pathology and recurrent blood transfusions [2, 5, 10]. Treatment consists of extraction of the excessive amount of iron from the body by phlebotomy [4, 5, 11]. When these phlebotomies are initiated before the development of irreversible symptoms and damage, HH patients have a normal life expectancy [11, 12]. Therefore, it is crucial that patients with HH are detected early in the course of the disease by measurement of their (elevated) serum iron parameters. However, these parameters are often not evaluated, as HH patients frequently present at ages between 50 to 60 years, with non-specific complaints, which are often ascribed to age related and common disorders [5, 13]. This non-specific presentation of the disorder reduces recognition of the disease and leads to high medical consumption and associated medical and non-medical costs [14, 15]. To enhance the awareness among physicians of HH in patients with these non specific symptoms and to improve the quality and effectiveness of the diagnostic pathway of HH, including the new HFE mutation analysis, a guideline for case detection and treatment of HFE-related HH was developed in our university hospital in 1998 by a multidisciplinary hemochromatosis study group.

In the present study we aimed to evaluate retrospectively i) physicians’ compliance with the diagnostic procedures, ii) the number of detected HH patients, iii) the correctness of the HH diagnoses, and iv) costs per detected patient, during a 2-year period before and after introduction of the clinical guideline.

METHODS

The multidisciplinary guideline was introduced in 1998 and contained recommendations to screen for HH when a patient presented with signs or symptoms as described in FIGURE 1. The guideline was developed in our university hospital by a multidisciplinary hemochromatosis study group. This group consisted of physicians of the departments of general internal medicine, haematology, rheumatology, clinical genetics, gastroenterology and clinical chemistry. International evidence based studies and expert opinion were translated into a guideline suitable for the local situation [8, 11, 16-20]. The guideline was introduced and explained during sessions held at the outpatient department of internal
medicine of our university medical hospital. After its introduction, the guideline was available on the intranet of our hospital server.

**Hereditary Hemochromatosis suspected in the presence of**
- Unexplained chronic fatigue for more than 6 months
- Elevation of liver enzymes without known cause
- Arthralgia of unknown origin existing longer than 6 month
- Poly-arthritis before the age of 50
- Cardiac rhythm disorders without diagnosis
- Unexplained heart failure
- Porphyria cutanea tarda
- Unexplained impotence and infertility
- Diabetes mellitus with elevated liver enzymes
- Unexplained iron storage in liver biopsy
- First-degree family members diagnosed with HFE-related PH

Serum transferrin saturation \( \geq 50\% \) and serum ferritin concentration \( \geq 280 \mu g/l \) at least on two occasions

Hereditary Hemochromatosis

Liver biopsy when ALAT or ASAT are at least twice the normal value

HFE-testing C282Y and H63D mutation

Hereditary Hemochromatosis (confirmed)

Treatment with phlebotomies withdrawal and maintenance phase

Family screening biochemical and genetic in first degree family members

**Figure 1** Flowchart on the diagnosis of HH.
Legend; see next page.
Impact of the introduction of a guideline on the targeted detection of hereditary hemochromatosis

FIGURE 1  Flowchart guideline for targeted detection of hereditary hemochromatosis (HH) as introduced in 1998.  

1 Provided that concomitant factors which influence the iron parameters, such as blood transfusions, iron supplementation, haemolytic anaemia, non-HH related liver disease and acute or chronic infection, could be excluded. Whenever doubt with respect to liver pathology, liver biopsy was recommended; 2 To exclude concomitant liver pathology or to obtain a prognosis for the condition of the iron overloaded liver; 3 For confirmation of the diagnosis HFE-related HH. C282Y homozygosity and C282Y/H63D compound heterozygosity confirm the diagnosis HFE-related HH. HFE-testing was also advised when the patient had first-degree relatives with known HFE related HH; 4 Two blood donations a year with control of the iron parameters once in 3 years, was recommended for C282Y homozygous and C282Y/H63D compound heterozygous first degree family relatives, of a HFE-related proband clinically expressing HH, who had not yet developed iron overload; 5 Genetic HH screening was recommended only when the proband was diagnosed with HFE-related HH; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase. 

According to the guideline, HH is diagnosed in these symptomatic patients, when the serum TS was equal or above 50% on at least 2 different occasions (one of which after overnight fasting), in combination with a SF concentration at least twice 280 µg/L or higher. For the diagnosis HH it was recommended to exclude other factors that are known to influence the iron parameters, such as blood transfusions, iron supplementation, haemolytic anaemia, (alcoholic) hepatitis, non-HH related liver disease and acute or chronic infections. Thus, a correctly diagnosed HH patient was defined as a patient with an elevation of both serum iron parameters in the absence of concomitant factors that influence iron parameters. 

After detection of the biochemical iron overload the guideline recommended to test for the C282Y and H63D-mutation in the HFE-gene, to determine whether the patient had an HFE-related form of HH (FIGURE 1). Genetic testing was also recommended for first-degree relatives of a symptomatic HFE-related HH proband. Liver biopsy was advised when a persisted elevation of both iron parameters was combined with a serum alanine aminotransferase (ALAT) or aspartate aminotransferase (ASAT) concentration more than twice the normal value, to either exclude concomitant liver pathology or to obtain a prognosis for the condition of the iron overloaded liver. When either HFE-genotype (C282Y homozygosity and C282Y/H63D compound heterozygosity) or liver biopsy confirmed the diagnosis of HH, family screening and treatment was recommended (FIGURE 1). The latter consisted of phlebotomy therapy in 2 phases. The first phase of weekly phlebotomies was meant to withdraw iron from the overloaded tissues, the second phase, of 2 to 8 phlebotomies a year, to maintain a low body iron level. For first-degree relatives that appeared to be C282Y homozygous or C282Y/H63D compound heterozygous and had not yet developed iron overload, a phlebotomy schedule consisting of 2 blood donations a year, similar to that used for regular blood donors, was recommended.
We retrospectively compared the diagnostic procedures for patients with features suggestive for HH visiting the outpatient department of internal medicine, between a period before (January 1995 to December 1996) and after (May 2000 to April 2002) introduction of the guideline. The choice of the latter period allowed sufficient time for uptake of the novel procedures from the in 1998 introduced guideline, whereas the first period was chosen before the discovery of the HFE-gene mutation [1].

**Patients**

Patients were selected for evaluation by using discharge diagnosis codes (classification system ICD-9-CM-codes) [21]. Some patients had received more than one diagnosis code. Discharge codes included were: “unexplained chronic fatigue for more than half a year”, “elevated liver enzymes or liver cirrhosis without explanation”, “unexplained arthralgia”, “diabetes mellitus”, “hereditary hemochromatosis” or “iron metabolism disorders” and “porphyria cutanea tarda”. Inclusion of diabetes mellitus patients was restricted to patients with concomitant elevation of liver enzymes (more than twice the upper limit of the reference value). Excluded were diabetes mellitus type 1 patients under the age of 35, patients suffering from chronic viral hepatitis, chronic alcohol abuse at the time of the study, cholestatic pathology and HH patients diagnosed with iron overload HH elsewhere. By means of an inventory form the following data were extracted from the medical records: serum TS, SF concentration, HFE-gene testing, liver biopsy, HH diagnosis and the presence of co-factors that might result in falsely elevated iron parameters (such as blood transfusions, iron supplementation, haemolytic anaemia, (alcoholic) hepatitis, acute or chronic infections, hepatic injury and end-stage liver disease).

**Compliance and Statistics**

Compliance of the physicians with the guideline was calculated by (number of guideline items followed) / (items followed + items not followed) x 100%. These items consisted of serum TS, SF concentration, HFE-gene testing and liver biopsy. For the period before guideline introduction the same items, except the HFE-gene testing, were scored.

Differences in diagnosis codes, gender, age and compliance scored before and after guideline introduction were tested for significance using a chi-square test.

**Costs**

The impact of the guideline introduction on resource utilization was assessed, taking into account direct medical costs only. The costs for diagnosing HH were approximated from laboratory costs (serum TS, SF and HFE-mutation detection) and the costs for ultrasound guided liver biopsy, with one-day hospital stay. For unit cost prices, national tariffs were used as proxies of actual resource
utilization, except for hospitalization, for which a standard cost price was used [22]. Volumes of tests used were derived from chart review. Costs per case of correctly diagnosed HH patients were calculated and expressed in Euro.

RESULTS

PATIENT SELECTION AND CHARACTERISTICS

During the 2 observation periods, a total of 9096 individual patients visited the outpatient department of internal medicine, providing us with 902 discharge diagnoses codes consistent with the possible presence of HH representing 878 patients, 422 patients from the period before and 456 patients from the period after guideline introduction (TABLE 1). In addition, 16 patients could not be included as their medical records were missing; 4 from the period before and 12 from the period after guideline introduction.

Three hundred fifty-two (40%) of the patients were male; 177 (41.9%) before and 175 (38.4%) after guideline introduction (TABLE 2). Overall, 561 (63.8%) of the patients were 50 years of age or older (TABLE 2).

DIAGNOSTIC ACCURACY

SERUM IRON PARAMETERS

In the period before guideline introduction, serum TS was measured in 29.7% (n=129) of all diagnosis codes. After introduction of the guideline, this percentage rose to 36.8% (n=172, p<0.05) (TABLE 3). The SF measurements were performed in 17.3% (n=75) of the patients before and in 71.8% (n=336, p<0.001) after introduction. This significant rise in SF measurements was observed for patients from all diagnosis codes, except for those with “liver cirrhosis of unknown origin”.

There was a pronounced rise in SF measurements for the diagnosis codes of “chronic fatigue of unknown origin” (from 10.9% (n=32) before to 74.7% (n=245, p<0.001) after), “diabetes mellitus with elevated liver enzymes” (from 18.0% (n=11) before to 44.9% (n=22, p<0.01) after) and “elevated liver enzymes of unknown origin” (from 29.6% (n=8) before to 73.1% (n=38, p<0.01) after). The hallmark test for the diagnosis HH, i.e. the combination of serum TS and SF measurement, also increased by guideline introduction from 12.2% (n=53) in the period before, to 29.5% (n=138, p<0.001) in the period after guideline introduction. This rise in combined measurement of serum TS and SF concentration was significant for all diagnosis codes, except for the small groups of patients diagnosed with “arthralgia of unknown origin” and “liver cirrhosis of unknown origin”.

79
### TABLE 1  Discharge diagnosis codes included in the study for both the periods before and after introduction of the guideline

<table>
<thead>
<tr>
<th>Diagnosis codes</th>
<th>Number of diagnosis codes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before introduction</td>
</tr>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Arthralgia, of unknown origin, &gt; 6 months</td>
<td>16</td>
</tr>
<tr>
<td>Chronic fatigue, of unknown origin, &gt; 6 months</td>
<td>294</td>
</tr>
<tr>
<td>Diabetes mellitus with elevated liver enzymes médical</td>
<td>61</td>
</tr>
<tr>
<td>Hemochromatosis or disturbed iron metabolism</td>
<td>6</td>
</tr>
<tr>
<td>Liver enzyme elevation of unknown origin</td>
<td>27</td>
</tr>
<tr>
<td>Liver cirrhosis of unknown origin</td>
<td>26</td>
</tr>
<tr>
<td>Porphyria Cutanea Tarda</td>
<td>4</td>
</tr>
<tr>
<td>Medical records not available</td>
<td>4</td>
</tr>
<tr>
<td>Total number of included diagnosis codes</td>
<td>434</td>
</tr>
<tr>
<td>Total number of included patients</td>
<td>422</td>
</tr>
</tbody>
</table>

ns, non significant; n.d., not determined; ¹ 100% = total of diagnosis codes included in that period; ² Significance of difference in number of patients included between the periods before and after implementation of the guideline; ³ All patients diagnosed with diabetes mellitus type 2 or diabetes mellitus type 1 after the age of 35 years. Liver enzymes were elevated when they were more than twice the normal values; ⁴ Cholestatic diseases, viral hepatitis and chronic alcohol abuse at time of diagnosis were excluded; ⁵ Diagnosis codes of patients’ medical records that were not available: before introduction 3 codes “diabetes of unknown origin” and 1 code “chronic fatigue”, after introduction 12 codes “adult onset diabetes mellitus of unknown origin”; ⁶ One patient could have more than 1 diagnosis code.

For all the discharge codes the absolute number of serum TS measurements was comparable to the absolute number of SF measurements after guideline introduction. Only the “chronic fatigue of unknown origin” defined group showed a striking difference between the 2 measurements; 87 serum TS measurements versus 245 SF measurements in the period after guideline introduction.
Effects of the introduction of a guideline on the targeted detection of hereditary hemochromatosis

**TABLE 2** Gender and age ( > 50 years) of the patients included

<table>
<thead>
<tr>
<th></th>
<th>Before implementation</th>
<th>After implementation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>422</td>
<td>48.1(^3)</td>
<td>456</td>
</tr>
<tr>
<td>Male</td>
<td>177</td>
<td>41.9(^1)</td>
<td>175</td>
</tr>
<tr>
<td>Female</td>
<td>245</td>
<td>58.1(^1)</td>
<td>281</td>
</tr>
<tr>
<td><strong>Age &gt; 50 years</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>268</td>
<td>63.5(^1)</td>
<td>293</td>
</tr>
<tr>
<td>Male</td>
<td>113</td>
<td>26.8(^1)</td>
<td>97</td>
</tr>
<tr>
<td>Female</td>
<td>155</td>
<td>36.7(^1)</td>
<td>196</td>
</tr>
</tbody>
</table>

n, number of patients; \(^1\) 100% = total included patients in the group before implementation (n=422); \(^2\) 100% = total included patients in the group after implementation (n=456); \(^3\) 100% = total included patients of the two periods together (n=878); \(^4\) Significance of difference between the periods before and after implementation of the guideline.

**HFE-MUTATION ANALYSIS**

HFE-gene mutation analyses were performed in 27 patients after protocol introduction (TABLE 4). According to the guideline HFE-testing was recommended for only 11 (40.7%) of these 27 patients (numbers 1-11); 9 of them had a combination of elevated serum TS and elevated SF concentration and 2 of them were screened within the framework of family screening. In 6 of these 11 patients the clinical diagnosis HH could be confirmed on follow-up, since both iron parameters remained elevated and no other explanation that could account for these elevated levels was found (TABLE 4). One of them had a non-HFE related form of HH, confirmed by the amount of iron withdrawn by phlebotomy to obtain normal serum iron parameters (number 6). In 3 of these 11 patients the HH diagnoses could not be confirmed (numbers 7-9); 1 patient’s liver biopsy contained no iron, 1 patient’s serum TS returned to normal levels when measured on a second occasion and 1 patient had normal TS levels, that alternated with high TS levels upon blood transfusion. The physicians were correct, not diagnosing HH in these 3 patients. The 2 patients, who were HFE-gene tested in the context of family screening (numbers 10 and 11), were falsely diagnosed as to be iron overloaded and treated as HH patients. The guideline recommended follow up of these patients and phlebotomize them only twice a year. The remaining 16 (of the 27) HFE-tested patients should not have been tested following the guideline, since only 1 of the 2 serum iron parameters was elevated. Moreover, 5 of these 16
patients (numbers 12-16) were incorrectly diagnosed with HH by the physicians, for some of them most likely based on their HFE-gene genotype only.

Three patients in the period after guideline introduction were not tested for the HFE-gene mutations despite their combination of elevated serum iron parameters. In 2 of these 3 patients serum iron parameters appeared to be temporarily influenced by blood transfusions. The remaining patient underwent a liver biopsy to exclude liver pathology. This liver biopsy revealed no iron.

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Diagnostic test use for serum iron parameters before and after guideline introduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum iron parameters</td>
<td>Diagnosis codes</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum TS</td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td></td>
</tr>
<tr>
<td>Combination of TS and SF concentration</td>
<td></td>
</tr>
</tbody>
</table>

Diagnostic test use is expressed as the percentage of the total number of diagnosis codes included in that period. Data are obtained from the medical records. Both the serum TS and the SF concentration were scored no more than once per diagnosis code.

TS, transferrin saturation; SF, serum ferritin; ¹Significance of difference in increase in serum iron parameter(s) between the periods before and after implementation of the guideline.

LIVER BIOPSY

Liver biopsies were taken for 60 diagnosis codes, representing 53 patients, 26 (49.1%) before and 27 (50.9%) after guideline introduction. In 49 of these 53 patients, the decision to perform a liver biopsy was based on a suspicion of concomitant liver disease. In 4 of these 53 patients, liver biopsy was performed in the absence of elevated liver enzymes or (probable) liver disease. Three of these latter 4 patients underwent liver biopsy before guideline introduction and the availability of the HFE-gene test. All 3 patients had elevated serum TS (>50%) and SF levels (>280 µg/l). The presence of an increased amount of iron in their liver biopsy (diagnosed by an independent pathologist) confirmed the diagnosis HH. The remaining fourth patient underwent his biopsy after guideline introduction in the presence of an elevated serum TS and in absence of an increased SF level. The liver biopsy revealed no increased amount of iron and HH was correctly excluded.
### TABLE 4  Characteristics of patients of whom HFE-gene analysis was performed or who were diagnosed with hereditary hemochromatosis according to the physicians or the guideline

<table>
<thead>
<tr>
<th>patient</th>
<th>evaluation period</th>
<th>serum iron parameters</th>
<th>HFE gene mutations</th>
<th>liver biopsy</th>
<th>HH diagnosis according to phys</th>
<th>guid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TS ≥ 50%</td>
<td>SF ≥ 280 µg/L²</td>
<td>C282Y</td>
<td>H63D</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>after</td>
<td>+</td>
<td>+</td>
<td>het</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>2-5</td>
<td>after</td>
<td>+</td>
<td>+</td>
<td>hom</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>after</td>
<td>+</td>
<td>+</td>
<td>neg</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>after</td>
<td>+</td>
<td>+</td>
<td>het</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>after</td>
<td>+</td>
<td>+</td>
<td>neg</td>
<td>micro nodular cirrhosis, Perls negative</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>after</td>
<td>+</td>
<td>+</td>
<td>neg</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>10-11</td>
<td>after</td>
<td>+</td>
<td>-</td>
<td>hom</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>after</td>
<td>+</td>
<td>-</td>
<td>hom</td>
<td>Perls negative</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>het</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>after</td>
<td>-</td>
<td>-</td>
<td>het</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>hom</td>
<td>n.d.</td>
</tr>
<tr>
<td>16</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>n.d.</td>
<td>+</td>
</tr>
<tr>
<td>17-18</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>steatosis, Perls negative</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>steatohepatitis, Perls negative</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>after</td>
<td>+</td>
<td>-</td>
<td>neg</td>
<td>Perls negative</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>22-23</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>24</td>
<td>after</td>
<td>-</td>
<td>-</td>
<td>het</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>after</td>
<td>-</td>
<td>-</td>
<td>neg</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>26</td>
<td>after</td>
<td>-</td>
<td>+</td>
<td>neg</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>after</td>
<td>-</td>
<td>-</td>
<td>neg</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>28-29</td>
<td>before</td>
<td>+</td>
<td>+</td>
<td>n.a</td>
<td>n.a</td>
<td>+</td>
</tr>
<tr>
<td>30</td>
<td>before</td>
<td>+</td>
<td>+</td>
<td>n.a</td>
<td>Perls positive, hepatocellular carcinoma</td>
<td>+</td>
</tr>
<tr>
<td>31</td>
<td>before</td>
<td>+</td>
<td>+</td>
<td>n.a</td>
<td>Perls positive</td>
<td>+</td>
</tr>
<tr>
<td>32</td>
<td>before</td>
<td>+</td>
<td>+</td>
<td>n.a</td>
<td>Perls positive, cirrhosis</td>
<td>+</td>
</tr>
<tr>
<td>33</td>
<td>before</td>
<td>n.a</td>
<td>n.a</td>
<td>n.a</td>
<td>Perls positive, cirrhosis</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: see next page.
Table 4: Characteristics of patients of whom HFE-gene analysis was performed or who were diagnosed with hereditary hemochromatosis according to the physicians or the guideline

TS, transferrin saturation; SF, serum ferritin; phys, Hereditary Hemochromatosis (HH) diagnoses according to the physician: diagnoses of iron overload based on clinical grounds and treatment started for HH; guid, HH diagnoses according to the guideline: HH diagnoses that should have been given according to the guideline; het, heterozygous; hom, homozygous; neg, negative; n.d., not determined; n.a., not available; Perls, Perls’ staining, Prussian blue reaction used to detect iron in a liver biopsy; 1 before, period before guideline implementation; after, period after guideline implementation; 2 transferrin saturation < 50% or serum ferritin < 280 µg/L; 3 transferrin saturation ≥ 50% or serum ferritin ≥ 280 µg/L; 4 phys., physician; guid, guideline; - no HH diagnosed, + HH diagnosed; 5 non-HFE related HH; 6 serum transferrin saturation that normalized when measured at a second occasion; 7 patient was a first-degree relative of an HFE-gene related HH patient; 8 HH diagnoses confirmed with either liver biopsy or number of phlebotomies.

NUMBER OF DETECTED PATIENTS

The introduction of the guideline led to an increase in diagnoses of HH, from 5 patients (1.2%) before to 13 patients (2.9%) after introduction of the guideline (Table 4). This increase, however, was not statistically significant. Phlebotomy treatment was started for all 18 patients.

The physicians’ diagnoses of iron overload appeared to be incorrect for 7 of the 13 patients, as at least 1 of the serum iron parameters was not elevated (patients 10-16, Table 4). Three of these patients were at risk of developing iron overload based on their C282Y homozygosity, though had not yet developed iron overload as their SF levels were normal (patients 10-12). All 3 were female and aged 41, 45 and 55 years, respectively. There was no over-diagnosis of HH before guideline introduction.

In total, we found 1 case of a missed HH diagnosis (patient 33, Table 4). This patient, included in the group before guideline introduction, was only diagnosed post mortem with HH by autopsy. During life, the patient was diagnosed with liver cirrhosis of unknown origin. No iron parameters had been measured.

For 9 patients with a combination of an elevated serum TS and SF, HH was not diagnosed. Three of these patients were included in the period before and 6 patients were included in the period after guideline introduction. One patient from the first period was diagnosed with porphyria cutanea tarda and transferred to another hospital before further diagnosis and treatment could take place. For all the remaining 8 patients the diagnosis HH was correctly excluded either based on clinical evidence (blood transfusions recently given or spontaneous normalization of iron parameters), or by a liver biopsy containing no increased amount of iron.
**COSTS**

The total cost associated with the detection of new HH patients before introduction of the guidelines amounted to 11,900 Euro. After the introduction, these costs rose to 15,600 Euro. When these costs were ascribed to patients correctly diagnosed with iron overload proven HH this resulted in 2,380 Euro per correctly diagnosed patient before and 2,600 Euro per correctly diagnosed patient after introduction of the guideline.

**DISCUSSION**

The introduction of the guideline for targeted HFE-related HH detection in the outpatient department of general internal medicine of our university hospital in 1998, led to an increased number of patients with complaints consistent with HH, that were tested for serum iron parameters (serum TS and SF). The number of HH diagnoses rose when compared to a period before guideline introduction. This rise, however, was not statistically significant. Shortcoming of the introduction of the guideline was the increase in the number of patients falsely diagnosed with HH.

The increase in both, serum TS and SF measurements, in the period after introduction of the guideline, was likely to result from the guideline introduction. This increase might have been positively influenced by more recently (after 1998) introduced guidelines at the department of internal medicine i.e. on “arthralgia” and on “liver cirrhosis” that incorporated the recommendations of the HH guideline of 1998. It should, however, be noted that despite these increased numbers of iron parameters measured after guideline introduction, still approximately 70% of the patients with complaints and signs consistent with HH were not tested for these parameters.

There was a remarkable difference in magnitude of the raise in serum TS and that in SF measurements in the diagnosis code group “chronic fatigue of unknown origin” after guideline introduction. This could be explained by the implementation of a guideline on “chronic fatigue”, at the outpatient department in 1999, which recommended only the measurement of SF, not combined with serum TS, to detect HH among patients with complaints suggestive for chronic fatigue.

Guideline compliance was also evaluated by the use of liver biopsies in the diagnosis of HH. According to the guideline liver biopsies should be used to exclude additional liver pathology or to obtain a prognosis for the condition of the iron overloaded liver. Before the discovery of the HFE-gene, liver biopsy was the gold standard for the confirmation of the diagnosis of hereditary iron overload. The compliance for the use of liver biopsies after the introduction of the guideline was good. Only 1 patient underwent a liver biopsy without elevation of both serum iron parameters or a possible liver disease.
The current study did not provide solid information on compliance with family screening for HH. However, the few notations made on this subject in the medical files suggested that physicians recommended the proband to inform his or her family on the necessity of clinical, biochemical and/or genetic screening for HH.

Medical costs due to diagnostic procedures for each accurately diagnosed patient were similar before and after guideline introduction. However, these costs do not include costs due to incorrect diagnoses: i.e. patients with HH missed or patients that are incorrectly diagnosed as having HH, nor does it include costs for treatment.

Compliance with the therapeutic aspects of the guideline was not thoroughly evaluated in the present study. However, it appeared that 3 homozygous C282Y patients of the 13 subjects diagnosed with HH were phlebotomized despite the absence of iron overload. For non-iron overloaded homozygous C282Y relatives of HH patients as well as for C282Y/H63D compound heterozygous relatives, the guideline recommended to perform preventive 2 blood donations twice a year. However, treatment of these non-iron overloaded patients is controversial and various treatment protocols have been proposed. Shortly after the discovery of the HFE-gene, therapeutic protocols for these patients, such as the protocol described here, were based on the assumption of a high penetrance of the HFE-gene mutation and advised: i) to perform phlebotomies several times a year to prevent iron accumulation, in order to maintain the SF level around 50 µg/l [5] and ii) 2 yearly blood donation, with control of the iron parameters once in 3 years (present guideline) [23]. Since evidence is accumulating that the phenotypic penetrance of homozygosity for the C282Y mutation is low, it is currently advised to only start treatment when iron overload is proven and control for clinical and biochemical manifestations of HH every 10-20 years [24].

A drawback of the guideline introduction was the incorrect diagnoses of iron overload for several patients after guideline introduction (n=7). This was mainly due to erroneous use and interpretation of the HFE-genotype (n=6). It appeared that HFE-testing was more often used than strictly indicated and that once the HFE-gene was genotyped, it dominated the results of the serum iron parameters and the liver biopsy. This dominant use and overestimation of the value of the HFE-genotype in the diagnostic process of HH might be attributed to misinterpretation of the huge amount of international literature since 1996 that suggested the clinical relevance of the C282Y-mutation due to the high clinical penetrance [3, 25-28]. Only recently, evidence accumulated that this penetrance of the homozygosity for the HFE-gene C282Y-mutation might be very low [9, 29-32]. But also, the fact that the diagnostic strategy of the present guideline lacks solid scientific evidence on its most crucial points (similar to the strategies throughout literature) [4, 10, 11, 33, 34] and is mainly based on professional expertise, might have decreased the compliance.
While awaiting for the calculation of the cost-effectivity of both population and cascade screening, most HH patients in the Netherlands are still detected by case detection, i.e. early detection of patients with HH that seek medical attention for symptoms suggestive for HH. According to a recent report of Cadet et al this strategy of targeted HH detection has been proven to be cost-effective [35]. Also the present study shows the potential cost-effectivity of targeted case detection in comparison to population screening as approximately 1 in 80 patients (1:84 (5 in 422) patients before, to 1:76 (6 in 457) patients after guideline introduction) have biochemical proven iron overload in comparison to 1 in 280 to 1 in 400 patients in the general population of northern European origin [36, 37]. A disadvantage of this targeted approach however, is the potential diagnostic delay in the course of the disease.

There were some limitations inherent to the study. First of all it was a retrospective study. This implicated that we had to interpret the thoughts of the physicians on the differential diagnosis of their patients’ complaints by looking at the diagnostic investigations performed on each patient. E.g. we cannot be sure that every SF or serum TS was performed in the light of HH diagnostics. Even more, elevated iron levels might have been missed and with this also potential patients with HH, with a risk for organ damage and early death [2, 26, 26]. It is not possible to give solid numbers for these patients not recognized to have HH for the current study. As 87.8% (n=381) of the diagnosis codes eligible for HH before and 70.5% (n=330) of these diagnosis codes after guideline introduction, were not evaluated for serum iron parameters, there could have been a fair number of missed HH diagnoses. However, for all those patients that were tested for both their serum iron parameters, we conclude that no eligible HH patients were incorrectly judged as being healthy. This also implicates that when HH was not diagnosed, despite the elevation of both serum iron parameters, this was done on correct clinical grounds, taking into account concomitant treatment or diseases, as the guideline recommended. Second limitation was the lack of control group. Therefore we cannot exclude that the rise in diagnostic procedures be explained by the increase of the number of physicians that adhered to a more ‘defensive’ kind of medicine by adding test and/or the general trend in time of an increased use of iron parameters (i.e. SF) in the last decade.

We conclude that due to a relatively low compliance to the guidelines: i) approximately 70% of the patients with complaints and signs consistent for HH were not tested for serum iron parameters and consequently patients with HH might have been missed and on the other hand ii) indication and interpretation of the genetic and iron parameters were misunderstood with as a result overdiagnosis of HH. The reason why physicians do not follow clinical practice guidelines are described by several groups [38-40]. One of them, Cabana et al, clearly reviewed and summarized the literature on this subject in 1999. This resulted in the recognition of a variety of barriers to guideline adherence, that include: i) knowledge (awareness, familiarity), ii) attitude: (agreement, self-efficacy, outcome
expectancy, ability to overcome the inertia of previous practice) and iii) external barriers to perform recommendations. We believe that in general these barriers all attributed partly to the less optimal compliance of the “hemochromatosis” guideline. We expect the most critical points of misuse and interpretation of iron parameters and genetic tests, observed in the present study, can be removed by a more professional evidence based development and dissemination of the guideline, that is combined by an appropriate education strategy on its most decisive aspects. In fact this approach is adopted by a multidisciplinary team of medical professionals in the Netherlands, that recently started with the development of an evidence based guideline under auspices of the Medical Scientific Board of the Dutch Institute for Healthcare CBO, in close cooperation with the Order of Medical Specialists. These guidelines will be evidence based and formulated along strict rules (www.cbo.nl). Among all, attention will also be paid to applicability in daily routine and the implementation strategy. Also, this team may learn from the shortcomings from the present study. It is expected that implementation of this guideline around early 2007 in medical practice throughout the Netherlands, will increase the compliance of the guideline, also on the decisive points.

We summarize that introduction of a guideline for targeted approach for HH screening increased the amount of diagnostic procedures appropriate for HH investigation. The number of detected HH patients increased non-significantly, at comparable costs per case detected, with a drawback of falsely positive HH diagnoses. The HH over-diagnoses reflected the difficulties in indication and interpretation of both serum iron parameters and HFE-genotypes. Therefore, the implementation strategy of the guideline should be improved to increase the awareness and to guarantee the compliance with the indication and interpretation of both the iron and genetic parameters.

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Impact of the introduction of a guideline on the targeted detection of hereditary hemochromatosis


First-degree relatives of C282Y homozygous probands with clinically detected hemochromatosis have increased morbidity compared to the general population. The HEmochromatosis FAmily Study (HEFAS)

Submitted


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ABSTRACT

Recent studies have suggested family screening as a sophisticated model for the early detection of HFE-related hereditary hemochromatosis (HH). However, until now, reports on the empirical demonstration of the relevance of an iron accumulation related health problem in families with this form of HH are lacking. We therefore compared the morbidity and mortality of first-degree family members of probands with clinically diagnosed HH with that of the general population. Between May 2003 and August 2005, data on iron parameters, morbidity and mortality were collected from 224 Dutch C282Y-homozygous probands with clinically overt HH and from 735 of their first-degree family members, all participating in the HEmochromatosis FAmily Study (HEFAS). These data were compared with results obtained from an age- and gender-matched normal population, collected by means of a population-based survey conducted in the Netherlands, the Nijmegen Biomedical Study (NBS). HEFAS and NBS participants filled out similar questionnaires on demographics, lifestyle factors, health, morbidity and mortality.

A significantly higher proportion of the HEFAS first-degree family members reported to be diagnosed with hemochromatosis related diseases, e.g. 45.7% vs. 19.4% of the matched normal population (McNemar test, p<0.001). Mortality among siblings, children and parents in the HEFAS population was similar to that in the relatives of the matched controls.

This is the first study to demonstrate that morbidity among first-degree family members of C282Y-homozygous probands previously diagnosed with clinically proven HH is significantly higher than that in an age- and gender-matched normal population. This justifies further studies on the value of a family-screening program.

INTRODUCTION

HFE-related hereditary iron overload is characterized by iron deposition in parenchymal organs [1, 2]. Early detection and phlebotomy prevent tissue damage and result in long-term survival similar to that seen in the general population [2-6]. Of northern European patients diagnosed with hereditary hemochromatosis (HH), 80% appear to be homozygous for the C282Y mutation in the HFE gene. The carrier frequency of this C282Y mutation in the general Caucasian population is estimated to be as high as 1 in every 10 persons [7]. Altogether, this would favour population screening to prevent disease-related morbidity. Recently, however, it was shown that not all C282Y-homozygous individuals develop symptoms of iron overload disease, questioning the penetrance of the HFE-gene mutations [8-11]. Therefore, family screening has been suggested, since this has proven efficacy in the detection of latent
homozygotes for frequent recessive mutations [12]. Until now, however, one important item in the World Health Organization (WHO) guidelines for screening for disease, published in 1968, has remained unanswered for the HH related family screening: Is HH in these families an important health problem? [13]. To date, to our knowledge there is no such a study that has extensively compared the morbidity and mortality in HFE-related HH families, with the morbidity and family-related mortality of a general, apparently healthy, population, whereas these outcomes are required to legitimate further research on the implementation of family screening.

Therefore, the objective of the present study is to compare self-reported morbidity among first-degree family members (FDFM) of C282Y-homozygous probands previously diagnosed with clinically manifest HH, with data obtained from age- and gender-matched controls from a normal population. Furthermore, the mortality rates among FDFM in these HH families, as reported by the HEFAS probands, are compared with the mortality among the FDFM of age- and gender-matched participants from the normal population. Notably, the study is observational and not designed to explain similarities and/or differences in outcomes of the morbidity and mortality rate for the 2 populations.

Data for the HH families were obtained from the HEmochromatosis FAmily Study (HEFAS), which was designed to collect clinical, biochemical, genetic and mortality data from Dutch C282Y-homozygous probands as well as from their first-degree relatives. All probands in the HEFAS had been previously diagnosed with symptomatic HFE-related HH. The controls were recruited from the Nijmegen Biomedical Study (NBS), a population-based survey conducted among 22,400 inhabitants of the Dutch city of Nijmegen in 2002-2003 [14].

STUDY POPULATION AND METHODS

HEFAS POPULATION

For this study, 280 probands diagnosed with symptomatic HFE-related HH from 5 different medical centres in the Netherlands were actively approached (Figure 1). The local medical ethics committees of each of these centres approved the study protocol before the start of the study. A total of 224 probands participated. They provided the HEFAS group with names and addresses of 972 first-degree relatives (defined in this study as biological parents, full siblings, and biological children), 18 years of age and older, of whom 735 met the inclusion criteria. Participants were included from May 2003 until August 2005.

INCLUSION

Only subjects who gave written informed consent were included in the study. Probands had to be at least 18 years old and to have been clinically diagnosed with C282Y-homozygous HH. The iron
overload had to be confirmed by initial serum ferritin (SF) and transferrin saturation (TS) values exceeding the thresholds of SF $\geq 280$ µg/L for men, SF > 80 µg/L for women under the age of 50, SF $\geq 180$ µg/L for women $\geq 50$ years and TS $> 50\%$ for both men and women. When either one or both pre-treatment plasma iron parameters were unavailable, the presence of iron overload was alternatively confirmed by previously performed liver biopsy (grade 3 iron deposition according to Sindram) or by the number of phlebotomies required to normalize SF (males $\geq 22$ phlebotomies = 5 g chelatable iron; females $\geq 13$ phlebotomies = 3 g chelatable iron) [1, 15].

**Figure 1** Flowchart of the invited and participating probands, the accompanying family members and their available data. Probands were classified as ‘defined proband’ when symptoms consistent with hereditary hemochromatosis, C282Y homozygosity and iron overload were present, confirmed by either the plasma iron parameters, iron levels in a liver biopsy or the number of phlebotomies. Laboratory data: iron parameters (transferrin saturation, serum ferritin) and HFE genotyping.

* Participating hospitals: Atrium Medical Centre, Heerlen/Brunssum, Radboud University Nijmegen Medical Centre, Rijnstate Hospital Arnhem, University Medical Centre Groningen, and University Medical Centre Utrecht.

**Questionnaires**
All participants were asked to fill out a questionnaire containing a large number of questions on demographics, lifestyle (smoking, use of alcohol, diet), health status, general medical history,
morbidity, medical history for HH, implementation of family screening, legal, psychological and societal implications, and family structure including familial mortality.

LABORATORY DATA
Data on the included probands and family members were extracted from the medical records of the participating hospitals. Information on iron parameters (TS and SF) and liver biopsy of the participants was obtained only at the time of diagnosis of HH or the time of screening for HH, whereas data on the HFE-genotype and especially on the number of phlebotomies were also collected at points in time after the initial investigations. When incomplete, the physician involved in the diagnosis and treatment of the participants was asked to provide the HEFAS team with these data. Finally, when the data remained deficient or the subjects declared that they had never been tested for HH, participants were offered counselling and blood testing by their general practitioner (GP).
Iron parameters for HEFAS were collected by several clinical laboratories. The TS and SF were quantified using validated, standardized, routine laboratory methods. HFE-genetic test results were obtained from routinely used genetic tests. The amount of iron in the liver biopsies was assessed semi-quantitatively [15].

THE NIJMEGEN BIOMEDICAL STUDY (NBS)
The Nijmegen Biomedical Study (NBS) is a population-based survey conducted among inhabitants of the city of Nijmegen in 2002-2003 [16]. Nijmegen is a town in the eastern part of the Netherlands with 156,000 inhabitants, approximately 87% of Caucasian descent. The aim was to obtain a representative sample of the normal population in the Netherlands that could be used as a universal control population for a wide range of medical studies. Randomly selected, age- and gender-stratified inhabitants of Nijmegen (n=22,452) were taken from the population registry and received an invitation to fill out a postal questionnaire on lifestyle and medical and family history that was comparable to the HEFAS questionnaire. Approval to conduct the NBS was obtained from the Institutional Review Board of the Radboud University Nijmegen Medical Centre. The response to the questionnaire was 41.7% (n=9371). In addition, 69.1% of these responders donated 30 ml of blood each for DNA isolation, serum and plasma (n=6473). Analysis of the plasma iron parameters was performed in the Departments of Clinical Chemistry and Chemical Endocrinology of the RUNMC.

STATISTICAL METHODS
In order to compare the demographics, lifestyle, blood loss, Body Mass Index (BMI), general health, medication and morbidity of the participants in the HEFAS to that of the general population, a one-to-one age- and gender-matched sample was randomly drawn from the 9371 participants in the NBS. In
both studies, the general mental health, physical functioning and vitality were measured according to
the SF-36 questionnaire [17] and fatigue was scored according to the shortened fatigue questionnaire
[18]. Each of these scores was rescaled from 0 to 100 and values below 65 were considered to
reflect diminished general mental health, diminished physical functioning, diminished vitality and
increased fatigue, respectively [17]. These cut-off points were used for further evaluation.
The number of hemochromatosis related medication use was calculated by counting for each person
the use of (1) analgesics, (2) anti-rheumatic drugs and of (3) cardiovascular medication (i.e. use of at
least one of the following: anti-hypertensive drugs, cardiovascular drugs and diuretics), resulting in a
score that ranged from 0 to 3. Similarly, the number of hemochromatosis related diseases was
calculated by counting for each person the presence of (1) diabetes mellitus, (2) liver disease, (3)
rheumatism, (4) fatigue (score ≥ 18) and (5) cardiovascular disease, resulting in a score that ranged
from 0 to 5. Hemochromatosis related medication use (yes, no) and hemochromatosis related
morbidity (yes, no), were used for further evaluation.

Comparison of iron parameters between HEFAS and NBS might be difficult, because of the large
between-centre variations, especially in the SF. We circumvented this potential setback and
compared HEFAS and NBS with regard to i) the percentage of elevated iron parameters using local
reference values for each of the participating laboratories, and ii) the absolute values of iron
parameters using data obtained in only one single laboratory, that of the RUNMC (ca. 25%). The
rationale for the choice of this laboratory is that the sera of all participants of the NBS were analyzed
at this location. Prior to the analysis, both the actual iron parameters and the BMI were transformed
logarithmically to improve skewness. Differences in the means of the logarithmically transformed data
between the HEFAS and the age- and gender-matched sample from the NBS were tested for
statistical significance using the t-test for paired data. The back-transformed mean differences with
the 95% confidence intervals are presented. These results demonstrate a relative increase in the
median of the HEFAS compared to the median of the NBS.

Differences in single proportions between the HEFAS probands and the age- and gender-matched
sample from the NBS were tested for statistical significance using McNemar’s test. The percentage
differences between the HEFAS and the NBS-sample were calculated together with the 95%
confidence intervals (95% CI) that take into account the matched pair design. Because p-values and
the corresponding confidence intervals are then univocally related (i.e., whether or not zero falls
within the confidence interval), presentation of both is redundant; therefore, only the differences with
the corresponding confidence intervals are presented here. As this study enhances a descriptive
study, no corrections for multiple comparisons have been performed.

The mortality within HEFAS families, as reported by the probands, was compared with the mortality
in the families of the matched NBS participants. Differences in mortality between the HEFAS and the
matched NBS sample were tested for statistical significance using Fisher's exact test, separately among parents, siblings and children. A two-tailed p-value < 0.05 was considered statistically significant. Analyses were performed using SAS version 8.2.

RESULTS

STUDY POPULATION

Of the 280 probands, 224 (80.0%) filled out the questionnaires and the informed consent forms (Figure 1). These 224 probands provided names and addresses of 972 FDFM, ≥ 18 years of age, of whom 735 (75.6%) were included. Figure 1 shows that 100% of the included probands gave permission for analysis of their laboratory results, whereas 17 (2%) family members did not approve retrieval of laboratory data from their records or agree to additional withdrawal of blood for laboratory tests if data were missing.

Table 1 shows the size and structure of the families of the included HEFAS probands. Twenty-four (10.7%) of the 224 probands that entered the study had more than 5 participating siblings, whereas 78 (34.8%) had no participating siblings. Four probands had more than 5 children included in the study, whereas 105 probands had no participating children. In total, this study involved 224 probands, 428 siblings, 241 children and 66 parents.

DEMOGRAPHICS

Table 2 shows the results of the self-reported demographics and lifestyle characteristics of the FDFM and the matched NBS participants. The median age at participation was 48 years (range: 18-97 years), and 56.7% of the participants were women. Because of the matched design these values are identical in both studies. In the HEFAS population, the percentage of FDFM with single households (with or without children) was 18.8%, which was significantly lower than the percentage with single households in the matched sample of the NBS population, which was 31.3%. The difference in the percentage of single households between the HEFAS and NBS populations (HEFAS% minus NBS%) was therefore -12.3%, with a 95%CI of -16.4% to -8.3% (Table 2). Furthermore, the number of participants with at least secondary education was significantly lower in the FDFM of the HEFAS population compared to the matched NBS participants (HEFAS% minus NBS%: -9.9%, 95%CI, -14.5% to -5.3%), while the percentage of participants with paid jobs was similar for both populations (HEFAS%-%NBS%: 2.8%, 95%CI, -4.8% to 10.3%). The HEFAS FDFM reported a significantly lower alcohol intake compared to the NBS controls (> 2 units alcohol/day, HEFAS%-%NBS%: -8.3%, 95%CI,
-13.2% to -3.4%). Yet, the smoking behaviour of both groups was similar (ever smokers, HEFAS%-NBS%: 0.8%, 95%CI, -0.4% to 5.7%). The number of participants that donated blood voluntarily was similar in both populations (TABLE 2).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Size and structure of the families of the HEFAS probands</th>
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<tbody>
<tr>
<td></td>
<td>Siblings</td>
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<td>Siblings</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Both parents</th>
<th>5</th>
<th>3</th>
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<th>1</th>
<th>3</th>
<th>3</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Father or mother</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>No parent</td>
<td>70</td>
<td>31</td>
<td>18</td>
<td>24</td>
<td>18</td>
<td>15</td>
<td>176</td>
</tr>
</tbody>
</table>

**GENERAL HEALTH, MEDICATION, MORBIDITY AND IRON PARAMETERS**

TABLE 3 summarizes the general health, medication, morbidity and iron parameters of the FDFM in the HEFAS population and the age- and gender-matched NBS participants. The median BMI of the HEFAS FDFM was slightly but significantly higher than that of the population-based controls of the NBS (HEFAS%-NBS%: 1.7%, 95%CI, 0.1%-2.4%). The HEFAS FDFM had significantly less hours of exercise during the week, and a significantly higher score on physical functioning and vitality, indicating a lower level of physical functioning and vitality [17]. Significantly more FDFM of the HEFAS population used antihypertensive drugs (HEFAS%-NBS%: 8.8%, 95%CI, 4.9%-12.6%), antirheumatic drugs (HEFAS%-NBS%: 5.9%, 95%CI, 2.9%-9.0%), cardiovascular drugs (HEFAS%-NBS%: 4.4%, 95% CI, 1.2%-7.5%), diuretics (HEFAS%-NBS%: 3.2%, 95%CI, 0.1%-6.2%) and lipid-lowering drugs (HEFAS%-NBS%: 3.0%, 95%CI, 0.1%-5.8%) compared to the NBS controls. Iron supplements were less frequently used by the HEFAS FDFM, than by the matched NBS participants (HEFAS%-NBS%: -9.0%, 95%CI, -12.7% to -5.2%).
Morbidity of the HEFAS population compared to the general population

<table>
<thead>
<tr>
<th>Characteristics of the first-degree family members of the HEFAS probands and of the age- and gender-matched NBS participants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HEFAS</strong></td>
</tr>
<tr>
<td><strong>Demographics</strong></td>
</tr>
<tr>
<td>age at participation (yr)</td>
</tr>
<tr>
<td>men</td>
</tr>
<tr>
<td>education (≥ secondary)</td>
</tr>
<tr>
<td>household (single with or without children)</td>
</tr>
<tr>
<td>paid job (≥ 32 hrs/week)</td>
</tr>
<tr>
<td><strong>Lifestyle</strong></td>
</tr>
<tr>
<td>alcohol (&gt; 2 units/day)</td>
</tr>
<tr>
<td>smoking (ever)</td>
</tr>
<tr>
<td><strong>Blood loss</strong></td>
</tr>
<tr>
<td>blood donation (never)</td>
</tr>
<tr>
<td><strong>Women</strong></td>
</tr>
<tr>
<td>menarche (≤ 12 year)</td>
</tr>
<tr>
<td>pregnancies ( &gt; 3)</td>
</tr>
</tbody>
</table>

CI, confidence interval, using the matched pair design; n.a., not applicable, because the first-degree family members of the HEFAS and the NBS participants are matched one-to-one by age and gender; * number of matched pairs with valid data; † the increase from HEFAS to NBS, using the matched pair design.

Cardiovascular disease, fatigue, hypercholesterolemia and hypertension were reported significantly more frequently by the FDFM of the HEFAS population than by the participants in the control population. Liver disease (HEFAS%-NBS%: 3.2%, 95%CI, 1.0%-5.4%), osteoporosis (HEFAS%-NBS%: 4.2%, 95%CI, 1.8%-6.6%) and rheumatism (HEFAS%-NBS%: 24.6%, 95%CI, 20.6%-28.6%) were also diagnosed significantly more frequently among the FDFM of the HEFAS population. In contrast, diabetes mellitus, infertility and thyroid disease were diagnosed with similar frequencies in both populations (TABLE 3).
### Table 3 General health, medication, morbidity and iron parameters in the first-degree family members of the HEFAS probands and of the age- and gender-matched NBS participants

<table>
<thead>
<tr>
<th></th>
<th>HEFAS</th>
<th>NBS</th>
<th>HEFAS - NBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>median (range) / n (%)</td>
<td>total</td>
</tr>
<tr>
<td><strong>Body Mass Index (kg/m²)</strong></td>
<td>717</td>
<td>24.9 (15.2 – 60.6)</td>
<td>718</td>
</tr>
<tr>
<td><strong>General health</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>exercise (≤ 1 hr/week)</td>
<td>415</td>
<td>109 (26.3%)</td>
<td>412</td>
</tr>
<tr>
<td>health (&gt; 2)‡</td>
<td>722</td>
<td>204 (28.3%)</td>
<td>733</td>
</tr>
<tr>
<td>physical functioning at this moment (≤ 23)§</td>
<td>684</td>
<td>339 (49.6%)</td>
<td>697</td>
</tr>
<tr>
<td>vitality last 4 weeks (≤ 17)¶</td>
<td>680</td>
<td>376 (55.3%)</td>
<td>701</td>
</tr>
<tr>
<td><strong>Medication used (yes)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>analgesics</td>
<td>627</td>
<td>321 (51.2%)</td>
<td>691</td>
</tr>
<tr>
<td>antihypertensive drugs</td>
<td>654</td>
<td>146 (22.3%)</td>
<td>690</td>
</tr>
<tr>
<td>antirheumatic drugs</td>
<td>601</td>
<td>63 (10.5%)</td>
<td>673</td>
</tr>
<tr>
<td>cardiovascular drugs</td>
<td>614</td>
<td>70 (11.4%)</td>
<td>681</td>
</tr>
<tr>
<td>diuretics</td>
<td>606</td>
<td>73 (12.0%)</td>
<td>683</td>
</tr>
<tr>
<td>folic acid</td>
<td>583</td>
<td>67 (11.5%)</td>
<td>655</td>
</tr>
<tr>
<td>lipid-lowering drugs</td>
<td>614</td>
<td>57 (9.3%)</td>
<td>682</td>
</tr>
<tr>
<td>iron supplements</td>
<td>718</td>
<td>87 (12.1%)</td>
<td>674</td>
</tr>
<tr>
<td>tranquillizers</td>
<td>618</td>
<td>148 (24.0%)</td>
<td>696</td>
</tr>
<tr>
<td>(multi-)vitamin preparations</td>
<td>613</td>
<td>221 (36.0%)</td>
<td>675</td>
</tr>
<tr>
<td>vitamin B complex</td>
<td>593</td>
<td>139 (23.4%)</td>
<td>668</td>
</tr>
<tr>
<td>vitamin C complex</td>
<td>601</td>
<td>197 (32.8%)</td>
<td>670</td>
</tr>
<tr>
<td>Hemochromatosis related medication</td>
<td>677</td>
<td>421 (62.2%)</td>
<td>708</td>
</tr>
</tbody>
</table>

CI, confidence interval, using the matched pair design; TS, transferrin saturation; SF, serum ferritin; * number of matched pairs with valid data; † the increase from HEFAS to NBS, using the matched pair design; ‡ 1: feeling good to 5: feeling bad; § 5: bad mental health to 30: good mental health, using the SF-36 health survey score [17]; ‼ 10: bad physical functioning to 30: good physical functioning, using the SF-36 health survey score [17]; ¶ 4: low vitality to 24: high vitality, using the SF-36 health survey score [17].
Table 3. Continued. General health, medication, morbidity and iron parameters in the first-degree family members of the HEFAS probands and of the age- and gender-matched NBS participants

<table>
<thead>
<tr>
<th>Morbidity</th>
<th>HEFAS</th>
<th>NBS</th>
<th>HEFAS - NBS</th>
<th>difference† (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>total</td>
<td>median (range) / n (%)</td>
<td>total</td>
<td>median (range) / n (%)</td>
<td>total*</td>
</tr>
<tr>
<td>anemia</td>
<td>620</td>
<td>99 (16.0%)</td>
<td>674</td>
<td>90 (13.4%)</td>
</tr>
<tr>
<td>cancer</td>
<td>621</td>
<td>35 (5.6%)</td>
<td>683</td>
<td>48 (7.0%)</td>
</tr>
<tr>
<td>cardiovascular disease</td>
<td>620</td>
<td>65 (10.5%)</td>
<td>685</td>
<td>28 (4.1%)</td>
</tr>
<tr>
<td>cerebrovascular accident</td>
<td>604</td>
<td>17 (2.8%)</td>
<td>675</td>
<td>9 (1.3%)</td>
</tr>
<tr>
<td>diabetes mellitus</td>
<td>620</td>
<td>25 (4.0%)</td>
<td>677</td>
<td>31 (4.6%)</td>
</tr>
<tr>
<td>fatigue (≥ 18)**</td>
<td>688</td>
<td>90 (13.1%)</td>
<td>683</td>
<td>54 (7.9%)</td>
</tr>
<tr>
<td>hypercholesterolemia</td>
<td>623</td>
<td>97 (15.6%)</td>
<td>684</td>
<td>80 (11.7%)</td>
</tr>
<tr>
<td>hypertension</td>
<td>648</td>
<td>184 (28.4%)</td>
<td>689</td>
<td>138 (20.0%)</td>
</tr>
<tr>
<td>infertility</td>
<td>604</td>
<td>22 (3.6%)</td>
<td>669</td>
<td>28 (4.2%)</td>
</tr>
<tr>
<td>liver disease</td>
<td>611</td>
<td>31 (5.1%)</td>
<td>669</td>
<td>17 (2.5%)</td>
</tr>
<tr>
<td>osteoporosis</td>
<td>612</td>
<td>47 (7.7%)</td>
<td>677</td>
<td>25 (3.7%)</td>
</tr>
<tr>
<td>rheumatism</td>
<td>638</td>
<td>199 (32.2%)</td>
<td>678</td>
<td>41 (6.0%)</td>
</tr>
<tr>
<td>surgery</td>
<td>722</td>
<td>499 (69.1%)</td>
<td>728</td>
<td>482 (66.2%)</td>
</tr>
<tr>
<td>thyroid disease</td>
<td>610</td>
<td>28 (4.6%)</td>
<td>671</td>
<td>30 (4.5%)</td>
</tr>
<tr>
<td>Hemochromatosis related diseases</td>
<td>652</td>
<td>298 (45.7%)</td>
<td>675</td>
<td>131 (19.4%)</td>
</tr>
</tbody>
</table>

Iron parameters††

| TS > 50% | 599 | 176 (29.4%) | 494 | 21 (4.2%) | 403 | 25.3% (20.5% ; 30.1%) |
| SF above normal (µmol/L)‡‡ | 487 | 198 (40.7%) | 409 | 106 (21.2%) | 333 | 16.5% (9.7% ; 23.3%) |
| TS (%)§§ | 207 | 38.4 (3.2 - 107.3) | 135 | 29.5 (4.8 - 97.7) | 135 | 37.1% (23.4% ; 52.5%) |
| SF (µmol/L)§§ | 207 | 119.0 (4.0 - 2308) | 137 | 93.9 (6.6 - 4737) | 137 | 32.4% (7.4% ; 63.1%) |

CI, confidence interval, using the matched pair design; TS, transferrin saturation; SF, serum ferritin; * number of matched pairs with valid data; † the increase from HEFAS to NBS, using the matched pair design; ‡ self reported diagnosis of morbidity made by a physician; ** 4: fatigue absent to 24: fatigue present, using the shortened fatigue questionnaire score [18]; †† at time of being tested for hereditary hemochromatosis; ‡‡ serum ferritin above the local upper reference value. §§ only participants tested in the Radboud University Nijmegen Medical Centre.
The number of hemochromatosis related medication use and the number of hemochromatosis related diseases in the first-degree family members of the HEFAS probands (black) and of the age- and gender-matched NBS participants (grey).

The iron parameters TS and SF were both significantly more elevated in the FDFM of the HEFAS probands compared to the matched NBS participants, with a difference between HEFAS and NBS for TS of 25.3% (95%CI, 20.5%-30.1%) and for SF of 16.5% (95%CI, 9.7%-23.3%) (TABLE 3). Similarly, the relative differences in the absolute values of TS and SF between the FDFM of the HEFAS and the matched NBS participants were 37.1% (95%CI, 23.4%-52.5%) and 32.4% (95%CI, 7.4%-63.1%), respectively, using only the samples measured in the RUMCN.
**Morbidity of the HEFAS population compared to the general population**

**Figure 2** shows both the number of hemochromatosis related medication use and diseases of the FDFM of the HEFAS population and the age- and gender-matched NBS participants. A significantly higher percentage of FDFM used hemochromatosis related medication, compared to the NBS participants, e.g. a difference between HEFAS and NBS of 13.3% (95% CI, 8.2%-18.4%). Similarly, a significantly higher percentage of FDFM reported to be diagnosed with one or more hemochromatosis related disease, e.g. a difference between HEFAS and NBS 25.7% (95% CI, 20.9%-30.5%).

**Mortality**

All 224 HEFAS probands provided data on the mortality of their first-degree relatives. The probands provided information on 427 parents, of whom 70.0% (n=299) had died by the end of our study (Table 4). These mortality figures did not differ significantly from the reported 73.6% (n=310) deceased parents of the 224 age- and gender-matched NBS participants (p=0.25). Similarly, the mortality among the siblings and children of the HEFAS families did not differ significantly from that of the NBS families.

**Table 4** Mortality among first-degree family members of both 224 HEFAS probands and age- and gender-matched NBS participants

<table>
<thead>
<tr>
<th></th>
<th>HEFAS</th>
<th>NBS</th>
<th>p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>deceased</td>
<td>n</td>
</tr>
<tr>
<td>parents</td>
<td>427</td>
<td>299 (70.0%)</td>
<td>421</td>
</tr>
<tr>
<td>siblings</td>
<td>709</td>
<td>93 (13.1%)</td>
<td>752</td>
</tr>
<tr>
<td>children</td>
<td>414</td>
<td>8 (1.9%)</td>
<td>372</td>
</tr>
</tbody>
</table>

Families, number of families reported by the HEFAS probands or the age- and gender-matched NBS participants; n, number of family members reported by the proband or the age- and gender-matched NBS participant, respectively; * p-value for difference in proportion between the HEFAS and the NBS group, using Fisher's exact test.

**Discussion**

Family screening can be a sophisticated model for screening for HH. To our knowledge, however, reports on the relevance of an iron overload related health problem in families with HH are lacking to date. Indeed, the present study is the first to reveal significantly more hemochromatosis related
diseases in the HEFAS population compared to the normal population. In contrast, the mortality in
the HEFAS population did not significantly differ from the normal population.

Earlier studies have already described fatigue, weakness and arthropathy as being related to HFE
gene mutations, whereas diabetes mellitus, abnormal liver function tests, impotence, hypothyroidism,
cardiomyopathy and hepatocellular carcinoma were mentioned as some of the more specific, organ-
related problems leading to increased morbidity and mortality [1, 2, 5]. Furthermore they described
that, if HH was diagnosed and treated in time, tissue damage could be prevented and a long-term
survival similar to that seen in the general population could be achieved [2-6]. Nevertheless, recent
studies claim that although some iron-overloaded patients with homozygosity for the C282Y mutation
in the HFE gene have a high and probably preventable morbidity, even more subjects with this
genotype had no symptoms at all [8-11]. Moreover, studies performed in several European countries
could not detect significant differences in the prevalence of untreated homozygotes among elderly
populations compared to younger groups [19-22]. This cast doubt on the adequacy of pre-
symptomatic population screening. Thus, family screening was suggested as it was thought to
increase the chances to find both C282Y homozygosity (theoretically existent in 25% of the siblings)
and an elevated penetrance of iron overload due to the sharing of iron metabolism modifying genes
or environmental factors with the clinically expressing proband. Indeed, focusing on FDFM of C282Y-
homozygous patients with clinically overt HH has been shown to produce a significant yield of
C282Y-homozygous individuals with high penetrance of iron accumulation, but with an unknown
increase of morbidity compared to the normal population [23-25]. McCune et al recently reported that
despite the presence of elevated iron parameters, the morbidity among C282Y-homozygous relatives
of probands identified by screening a group of blood donors was similar to that of C282Y-
homozygous relatives of probands presenting as patients [26]. Assuming that the C282Y
homozygous blood donors had less morbidity than the probands of identical genotype presenting
clinically, this doubted the contribution of the higher penetrance of iron overload within HFE-mutated
families and therefore the significance of family screening. In the present study, however, we
demonstrated that first-degree relatives of patients with clinically overt HFE-related HH, do have a
significantly higher morbidity in comparison to the general population, thus supporting the additive
value of family screening. Nonetheless, the present study was not designed to clarify the factor(s)
responsible for the morbidity difference between both HEFAS and NBS populations, e.g. HFE-
genotype, iron metabolism modifying genes or environmental factors.

A remarkable finding in this study is the discrepancy between the higher morbidity and similar
mortality among the FDFM of the HEFAS probands compared to the matched NBS population.
Several explanations can be given. First of all, HEFAS family members as well as their general
practitioners may be more aware of the symptoms typical for HH, leading to an advantage in
diagnosis and treatment [23]. Secondly, the age of the C282Y homozygous siblings (mean 54 yrs, interquartile range Q1-Q3 47yrs-62yrs) might be too low for HFE-related mortality and the study might also comprise too few C282Y homozygous parents to influence the mortality differences between both parental populations. Next to this, other confounding factors that were not measured may have influenced the comparative mortality. It has, for instance, been suggested that C282Y polymorphism may protect against several infectious agents, either by the synthesis of a dysfunctional HFE protein as target receptor for infectious agents, by lowering the iron levels inside macrophages and so inducing resistance to ferrophilic micro organisms, or by altering immunological processes, all leading to an advantage in survival [3, 27-30]. More recent investigations have demonstrated that non-transferrin-bound iron in the sera of homozygotes and even heterozygotes for the C282Y mutation promoted the adhesion of monocytes to endothelial cells, which may be another advantage for immune defence [31]. Furthermore, the HFE gene mutations may provide a survival advantage by ameliorating the iron deficiency seen in another common HLA-defined condition, such as celiac disease [32]. Meanwhile, however, questions on the survival advantage of HFE polymorphism remain.

It should be noted that our study comprehends a self-reporting questionnaire. Therefore, to diminish a potential registration bias, the questionnaires for both HEFAS and NBS participants were similar, the participants were asked to report diseases as diagnosed by their physicians and the fatigue and general health questions were scored by validated questionnaires.

This study was not designed to clarify the causative factor in the observed morbidity differences. It is evident, however, that HEFAS relatives have a higher possibility of being homozygous for the C282Y mutation compared to the normal population. It is also likely that the risk of a similar predisposition, which enhances the biochemical and clinical penetrance of C282Y homozygosity, is highest among first-line relatives of overt HH patients. Yet, the exact nature of this predisposition is still largely unknown (reviewed in Swinkels et al [33]).

Taken together, we are the first to demonstrate that the morbidity among first-degree relatives of probands with clinically overt HFE-related HH is significantly higher than in the normal population. This finding justifies further evaluation of the value of family screening in the early detection of HH and challenges us to find environmental or genetic factors that increase the risk for iron overload.

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Determinants for the phenotypic expression of iron overload in Dutch families with HFE-related hemochromatosis. The HEmochromatosis FAmily Study (HEFAS)

Submitted

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ABSTRACT

Recently family screening is suggested to prevent morbidity and mortality in families of patients with clinically detected hereditary hemochromatosis (HH). However, the clinical penetrance of the HFE-gene mutations is discussed and it is unknown which family members are at risk for the accumulation of iron and should be screened.

The aim of this study was to identify factors that predict the accumulation of iron in first-degree family members of probands with clinically detected HH. Between May 2003 until August 2005 data on HFE-genotype, iron parameters, demographics, lifestyle factors and health, were collected from 224 Dutch C282Y homozygous patients with clinically diagnosed HH and 735 of their first-degree family members, all participating in the HEmochromatosis FAmily Study (HEFAS).

The risk for iron accumulation was significantly higher in C282Y homozygous first-degree family members, compared to non-HFE mutation carriers, odds ratio (OR) 59.87 (95% Confidence Interval (CI) 21.22-168.84). Other determinants for iron accumulation were compound heterozygosity (OR 5.3, 95%CI 2.26-12.46), severity of iron accumulation in the proband (OR 1.06, 95%CI 1.02-1.10) and age at testing for serum ferritin (OR 1.03, 95%CI 1.01-1.05). Of the environmental factors supplemental iron intake was most indicative for iron accumulation (OR 2.16, 95%CI 1.10-4.24), while a low BMI showed a protective effect (OR 0.25, 95%CI 0.07-0.94).

This study shows that a well-defined group among the first-degree family members of C282Y homozygous patients with clinically detected HH is at risk for iron accumulation. This outcome will be helpful achieving an optimal strategy for future family screening programs.

INTRODUCTION

HFE-related hereditary hemochromatosis (HH) is an autosomal recessive disease characterized by a progressive abnormal deposition of iron in liver, joints, heart, pancreas and other vital organs, resulting in e.g. liver cirrhosis, arthralgia, cardiac failure, rhythm disorders and diabetes mellitus [1-3]. The subsequent increase in organ failure and early death is prevented through removing the accumulated iron by phlebotomy before irreversible organ damage occurs [2, 4]. We do know that the homozygous C282Y HFE-gene mutation is highly correlated to iron accumulation in diverse organs. To prevent HH related disease and early death, screening of those at risk for the development for iron overload is warrantable. However, as the penetrance of the C282Y homozygous mutation is by far not 100%, more parameters than only HFE-gene mutations are needed to separate, during screening, those who are at risk for iron overload from those who are not [2, 5-9].
The development of iron overload is well described by a five grade scale [10]. It shows that the first sign of an increased iron accumulation is an elevation of the serum transferrin saturation (TS) [10]. Indeed, reflecting a pathological iron homeostasis, in 50-100% of the C282Y homozygous population the measured TS is found to be elevated, depending on the cut-off value used [11, 12]. Subsequently, iron accumulates in the organs during lifetime, which is reflected by a gradual raise in serum ferritin (SF) [13, 14]. The SF of the C282Y homozygous individuals is mostly higher than that of the normal population during whole lifetime, however, its level is strongly influenced by age, gender and interfering diseases, making it more difficult to interpret its value as reflection of the amount of accumulated iron [15-18]. Even more, there are indications that the clinical penetrance of the HFE-gene mutations is influenced by other, not yet well described, environmental, life style or genetic factors [2, 19, 20]. So for diagnosing HFE-related HH in early stage, as one would like to achieve during screening, next to information on the HFE-gene mutation, information is necessary on TS, SF, and factors determining the iron accumulation (SF) during the years.

Individuals with the highest chance of developing HFE-related iron overload are most likely the first-degree family members (FDFM) of clinically diagnosed C282Y homozygous probands. This because they have an elevated genetical risk for being C282Y homozygous (25% for siblings) and because they are prone to share similar unknown environmental, life style and modifiable genetic factors, leading to a high clinical penetrance of the HFE-gene mutation, and consequently increased iron accumulation and more severe illness.

In the present study gender, age, HFE-gene mutations, iron parameters, life style factors and family relation to the clinically detected C282Y homozygous proband (parent, child or sibling) of FDFM are studied, to detect determinants for iron overload, as reflected by SF. Next to this, an interfamilial comparison of the accumulated amount of iron was performed, to detect the possibility of other, not identified (genetic) factors. Taken altogether, the identification of factors that predict for iron overload in relatives of patients with HFE-related HH will be helpful for the optimization of screening strategies in these families.

**STUDY POPULATION AND METHODS**

**Definition of HH in the present study**

With HH in this study is meant HFE-related hemochromatosis with iron overload and related disease. With HH is not meant the HFE-gene mutation alone.
HEMOCHROMATOSIS FAMILY STUDY (HEFAS) POPULATION

A total of 224 probands participated. They provided the HEFAS group with names and addresses of 972 FDFM, 18 years of age and older, of whom 735 met the inclusion criteria. FDFM are defined in this study as biological parents, full siblings, and biological children. The FDFM of one proband, form, together with this proband, one family. Families were recruited from May 2003 until August 2005. The local medical ethics committees of the participating centres (Atrium Medical Centre Heerlen/Brunssum, Radboud University Nijmegen Medical Centre, Rijnstate Hospital Arnhem, University Medical Centre Groningen and University Medical Centre Utrecht) approved the study protocol before the start of the study.

INCLUSION CRITERIA

Only subjects who gave written informed consent were included in the study. Probands were at least 18 years old and clinically diagnosed with C282Y-homozygous HH. HFE-related accumulation of iron was confirmed by initial transferrin saturation (TS) and serum ferritin (SF) values exceeding the reference value thresholds; TS > 50% for both men and women, SF ≥ 280 µg/L for men, SF > 80 µg/L for women under the age of 50, and SF ≥ 180 µg/L for women ≥ 50 years, or corresponding values for SF depending on the reference values of the laboratories. When either one or both pre-treatment serum iron parameters were unavailable, the presence of iron overload was alternatively confirmed by previously performed liver biopsy (grade 3 iron deposition according to Sindram) or by the number of phlebotomies required to normalize SF (males ≥ 22 phlebotomies = 5 g chelatable iron; females ≥ 13 phlebotomies = 3 g chelatable iron) [2, 21].

QUESTIONNAIRES

All participants were asked to fill out a questionnaire compromising a large number of questions on demographics, lifestyle (smoking, use of alcohol, diet), health status, general medical history, morbidity, medical history for HH, and family structure.

LABORATORY DATA

Data on the included probands and family members were extracted from medical records of the participating hospitals or acquired from the physicians involved in diagnosis and treatment of the patients. Information on iron parameters (TS and SF) and liver biopsy of the participants was obtained at the time of diagnosis or screening of HH, whereas data on HFE-genotype and especially on the number of phlebotomies were also collected at points in time after the initial investigations. Whenever the participants declared never been tested for HH, counselling and blood testing by their general practitioner was offered.
The TS and SF were quantified using validated, standardized, routine laboratory methods. HFE-genetic test results were obtained from routinely used genetic tests.

**STATISTICAL METHODS**

In this study we aimed at identifying FDFMs, of probands with clinically detected HFE-related HH, at risk for iron accumulation from those not at risk. For this purpose, elevated TS was defined as TS above 50%, elevated SF as SF above the gender- and calendar time-specific local laboratory reference values. It may be noted that some of the laboratories also used different reference values for premenopausal and post-menopausal women. In some cases where the SF reference values were not available, the 67th percentile of all reference values was used. Furthermore, different reference values for premenopausal and postmenopausal women were taken into account when provided by the laboratories.

In the following analyses the probands were excluded. Univariate logistic regression was used to study the ability of environmental, life habits and genotype variables to discriminate FDFM with elevated iron variables from FDFM with non-elevated iron variables, for each variable separately. The dependent variables were elevated TS and elevated SF, respectively. The crude odds ratios (ORs) with 95% confidence intervals (CIs) are presented.

Multivariate logistic regression with stepwise selection procedures was used to identify variables that contributed independently to the risk of elevated iron variables, either next to genotype or next to family-degree. In this way, genotype models and family-degree models were studied. Again, the dependent variables were elevated TS and elevated SF, respectively.

Possible iron accumulation determining variables used in the selection procedure were gender, age at testing, BMI, iron supplements, alcohol use, familial iron severity. The familial iron severity was defined as the value of TS of the proband in case elevated TS was studied and the value of SF of the proband divided by the reference value in case of elevated SF. The adjusted ORs with 95%CIs of the final model are presented. The total R-square is presented to indicate the total percentage explained variance in the outcome and the area under the curve (AUC) of the receiver operating characteristic (ROC) curve is presented as measure of predictive discrimination.

The fit of model is visualized in a figure that shows the estimated and observed iron overload. The results of the final genotype model are also used to estimate the probability of elevated ferritine levels (with 95% CI) of C282Y homozygous family members by gender, age, BMI, use of iron supplements and familial iron severity. Note that this estimates the penetrance (of “elevated ferritine values”), including modifying factors.

All statistical analyses were performed using SAS version 8.2.
RESULTS

DEMOGRAPHICS AND CLINICAL CHARACTERISTICS
The family members of the HEFAS population consisted of 224 probands, 428 siblings, 241 children and 66 parents (TABLE 1). The percentage male participants was slightly higher in the group of probands, 62.5% (n=140), but lower among the siblings, 46.3% (n=198) and children 40.7% (n=98). The percentage male participants of the parents was even lower (33.3%, n=22), reflecting the higher age of survival of the women. The ages of the participants varied from a median of 56 years for probands, 54 years for siblings, 32 years for children, to finally 70 years for parents.

GENOTYPE CHARACTERISTICS AND IRON PARAMETERS
TABLE 1 also presents the genotype characteristics of the HEFAS population. 100% of the probands were C282Y homozygous (by definition), compared to 29.9% (n=110) of the siblings, 5.7% (n=11) of the children and 2.2% (n=1) of the parents, whereas C282Y heterozygosity was determined in 39.7% (n=146), 78.1% (n=150) and 78.3% (n=36) of the siblings, children and parents, respectively.

The mean TS found of the probands was 86.8% (Q1-Q3; 74.0%-96.3%), which was significantly higher than the mean TS of the other family groups, p<0.0001. The same is true for the TS >50%. The probands also revealed significantly higher values of absolute SF compared to the other FDFM, p<0.0001. Notice that not in all probands both the TS value and the SF value were elevated. In that case however, the excess of iron was confirmed either by liver biopsy or by phlebotomy.
In the homozygous HEFAS population TS >50% was found in 93.2% (n=192) of the probands, 86% (n=84) of the siblings, 56% (n=5) of the children and 100% (n=1) of the parents. For an elevated SF these percentages amounted to 86.3% (n=183) of the parents, 80% (n=84) of the siblings, 22% (n=2) of the children and 100% (n=1) of the parents.

POTENTIAL FACTORS INFLUENCING TS AND SF LEVELS
TABLE 2 shows the odds ratio of the possible predisposing iron storage factors. As expected the genotype had a profound influence on the TS and SF levels. Being C282Y homozygous was correlated with a significantly raised risk for both elevated TS and elevated SF compared to being WT/WT (OR 80.29, 95% CI 36.83 to 175.03 and OR 22.50, 95%CI 12.22 to 41.42, respectively). Similarly, being compound heterozygous (C282Y/H63D) gave a raised risk for elevated TS and iron accumulation with OR 4.84, 95%CI 1.96-11.97 and OR 4.02, 95%CI 1.84-8.80, respectively.
### TABLE 1  Characteristics of the HEFAS population by family degree

<table>
<thead>
<tr>
<th></th>
<th>Probands</th>
<th>Siblings</th>
<th>Children</th>
<th>Parents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total</td>
<td>total</td>
<td>total</td>
<td>total</td>
</tr>
<tr>
<td></td>
<td>median (Q1-Q3) / n (%)</td>
<td>median (Q1-Q3) / n (%)</td>
<td>median (Q1-Q3) / n (%)</td>
<td>median (Q1-Q3) / n (%)</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>224</td>
<td>428</td>
<td>241</td>
<td>66</td>
</tr>
<tr>
<td>age at participation (yr)</td>
<td>224</td>
<td>428</td>
<td>241</td>
<td>66</td>
</tr>
<tr>
<td>education (≥ secondary)</td>
<td>212</td>
<td>395</td>
<td>233</td>
<td>61</td>
</tr>
<tr>
<td>paid job (≥ 32 hrs/week)</td>
<td>78</td>
<td>178</td>
<td>160</td>
<td>4</td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>215</td>
<td>417</td>
<td>238</td>
<td>62</td>
</tr>
<tr>
<td>Lifestyle</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>smoking (ever)</td>
<td>220</td>
<td>421</td>
<td>241</td>
<td>65</td>
</tr>
<tr>
<td>alcohol &gt; 2 units/day</td>
<td>189</td>
<td>374</td>
<td>204</td>
<td>50</td>
</tr>
<tr>
<td>Iron supplements</td>
<td>218</td>
<td>416</td>
<td>240</td>
<td>62</td>
</tr>
<tr>
<td>Meat consumption (&gt;200 grid)</td>
<td>214</td>
<td>398</td>
<td>230</td>
<td>59</td>
</tr>
<tr>
<td>Blood donation (no)</td>
<td>212</td>
<td>409</td>
<td>239</td>
<td>57</td>
</tr>
<tr>
<td>Menarche ≤ 12 year)**</td>
<td>80</td>
<td>223</td>
<td>141</td>
<td>39</td>
</tr>
<tr>
<td>Pregnancies (&gt; 3)**</td>
<td>67</td>
<td>196</td>
<td>84</td>
<td>41</td>
</tr>
<tr>
<td>Genotype</td>
<td>224</td>
<td>368</td>
<td>192</td>
<td>46</td>
</tr>
<tr>
<td>C282Y/C282Y</td>
<td>224 (100%)</td>
<td>110 (29.9%)</td>
<td>11 (5.7%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>C282Y/H63D</td>
<td>0</td>
<td>19 (5.2%)</td>
<td>27 (14.1%)</td>
<td>8 (17.4%)</td>
</tr>
<tr>
<td>C282Y/WT*</td>
<td>0</td>
<td>146 (39.7%)</td>
<td>150 (78.1%)</td>
<td>36 (78.3%)</td>
</tr>
<tr>
<td>WT/WT†</td>
<td>0</td>
<td>93 (25.3%)</td>
<td>4 (2.1%)</td>
<td>1 (2.2%)</td>
</tr>
<tr>
<td>Iron parameters§</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TS (%)</td>
<td>206</td>
<td>361</td>
<td>189</td>
<td>49</td>
</tr>
<tr>
<td>TS &gt; 50%</td>
<td>206</td>
<td>361</td>
<td>189</td>
<td>49</td>
</tr>
<tr>
<td>SF (µmol/L)</td>
<td>212</td>
<td>366</td>
<td>191</td>
<td>48</td>
</tr>
<tr>
<td>SF above normal (µmol/L)§</td>
<td>212 183 (86.3)</td>
<td>366 133 (36.4)</td>
<td>191 32 (16.8)</td>
<td>48 13 (27.1)</td>
</tr>
</tbody>
</table>

Legend; see next page.
Compared to the other FDFM, the probands showed the highest chance on elevated TS (OR 53.47, 95%CI 22.15-129.11) and elevated SF (OR 16.99, 95%CI 8.05-35.88), which is not surprising taken into account the genotype distribution over the probands compared to the FDFM. Being a male also significantly raised the risk of elevated TS (OR 2.04, 95%CI 1.54-2.70), and of elevated SF (OR 1.91, 95%CI 1.45-2.54). The age at testing was only identified as a significant risk factor for an elevated SF, as was having an abnormal BMI and higher meat consumption. Alcohol consumption (>2 units/day) and blood donation showed no influence on the iron parameters in this univariate analysis. The intake of iron supplements lowered the risk of elevated TS (OR 0.59, 95%CI 0.37-0.95), whereas women who had >3 pregnancies, had a higher risk of elevated TS (OR 1.51, 95%CI 0.96-2.36) and of elevated SF (OR 1.59; 95%CI 1.01-2.48).

TABLE 3 shows the adjusted odds ratios of the genotype model of elevated TS and of elevated SF. C282Y Homozygosity and compound heterozygosity increased significantly the risk of elevated TS compared to WT/WT (OR 59.87, 95%CI 21.22-168.94 and OR 4.89, 95%CI 1.67-14.35, respectively). Interestingly, BMI > 30 kg/m² independently diminished the risk for TS elevation. Both the R-square and the AUC of this genotype model are large. In total 44.6% (R-square) of the variance in elevated TS could be explained by the selected variables and the discriminatory power is 83.2% (AUC). For comparison an AUC equal to 50% is equivalent to random guessing.

As with elevated TS the risk for elevated SF was significantly increased by possessing the C282Y homozygous or compound heterozygous genotype compared to WT/WT (OR 20.87, 95%CI 9.74-44.75 and OR 5.30, 95%CI 2.26-12.46, respectively). Furthermore, the familial iron severity was predictive for an elevated SF (OR 1.06, 95%CI 1.02-1.10), as was the age of testing (OR 1.03, 95%CI 1.01-1.05). A BMI <20 kg/m² was protective for the risk of elevated SF, whereas the intake of iron supplements induced the risk. Noteworthy is that gender is not statistical significant in this multivariable logistic regression analysis. This may be indicative for the idea that gender is not an independently risk factor of an elevated SF that is based on age and gender specific reference values. The R-square and AUC are similar to those of the genotype model of elevated TS, 45.3% and 84.7%, respectively.
### TABLE 2
The crude odds ratios with 95% confidence intervals of increased transferrin saturation and serum ferritin values among all family members, using univariate logistic regression analysis

<table>
<thead>
<tr>
<th></th>
<th>Elevated TS odds ratio (95%CI)</th>
<th>Elevated SF odds ratio (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C282Y/C282Y</td>
<td>80.29 (36.83-175.03)</td>
<td>22.50 (12.22-41.42)</td>
</tr>
<tr>
<td>C282Y/H63D</td>
<td>4.84 (1.96-11.97)</td>
<td>4.02 (1.84-8.80)</td>
</tr>
<tr>
<td>C282Y/WT*</td>
<td>1.73 (0.82-3.66)</td>
<td>0.81 (0.43-1.51)</td>
</tr>
<tr>
<td>WT/WT†</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td><strong>Family degree</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>probands</td>
<td>53.47 (22.15-129.11)</td>
<td>16.99 (8.05-35.88)</td>
</tr>
<tr>
<td>siblings</td>
<td>1.92 (0.93-3.98)</td>
<td>1.54 (0.79-3.00)</td>
</tr>
<tr>
<td>children</td>
<td>1.29 (0.60-2.79)</td>
<td>0.54 (0.26-1.14)</td>
</tr>
<tr>
<td>parents</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td><strong>General characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>2.04 (1.54-2.70)</td>
<td>1.91 (1.45-2.54)</td>
</tr>
<tr>
<td>age at testing</td>
<td>1.00 (1.00-1.02)</td>
<td>1.03 (1.02-1.04)</td>
</tr>
<tr>
<td>education (≥ secondary education)</td>
<td>0.95 (0.69-1.31)</td>
<td>1.10 (0.80-1.51)</td>
</tr>
<tr>
<td>paid job (≥ 32 hrs/week)</td>
<td>1.00 (0.65-1.53)</td>
<td>1.04 (0.68-1.60)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.99 (0.95-1.02)</td>
<td>1.08 (1.04-1.12)</td>
</tr>
<tr>
<td>Alcohol &gt; 2 units/day</td>
<td>0.76 (0.54-1.01)</td>
<td>0.80 (0.59-1.12)</td>
</tr>
<tr>
<td>Iron supplements</td>
<td>0.59 (0.37-0.95)</td>
<td>0.85 (0.54-1.33)</td>
</tr>
<tr>
<td>Meat consumption (&gt;200 g/d)</td>
<td>0.85 (0.63-1.14)</td>
<td>1.51 (1.12-2.04)</td>
</tr>
<tr>
<td>Blood donation (no)</td>
<td>0.79 (0.56-1.10)</td>
<td>1.11 (0.79-1.56)</td>
</tr>
<tr>
<td>Menarche (≤ 12 year)</td>
<td>0.83 (0.44-1.56)</td>
<td>1.12 (0.60-2.10)</td>
</tr>
<tr>
<td>Pregnancies (&gt; 3)</td>
<td>1.51 (0.96-2.36)</td>
<td>1.59 (1.01-2.48)</td>
</tr>
</tbody>
</table>

TS, transferrin saturation; SF, serum ferritin; * C282Y/WT includes C282Y/WT, C282Y/unknown; † WT/WT includes H63D/H63D, H63D/WT, WT/WT; ** only women.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Elevated TS OR_{adj} (95%CI)</th>
<th>Elevated SF OR_{adj} (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C282Y/C282Y</td>
<td>59.87 (21.22-168.94)</td>
<td>20.87 (9.74-44.75)</td>
</tr>
<tr>
<td>C282Y/H63D</td>
<td>4.89 (1.67-14.35)</td>
<td>5.30 (2.26-12.46)</td>
</tr>
<tr>
<td>C282Y/WT*</td>
<td>1.75 (0.72-4.27)</td>
<td>0.99 (0.50-1.93)</td>
</tr>
<tr>
<td>WT/WT**</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>1.66 (0.08-1.66)</td>
<td>1.34 (0.82-2.17)</td>
</tr>
<tr>
<td>female</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Familial iron severity</td>
<td>1.00 (0.99-1.02)</td>
<td>1.06 (1.02-1.10)</td>
</tr>
<tr>
<td>Age at testing</td>
<td>1.00 (0.98-1.01)</td>
<td>1.03 (1.01-1.05)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>0.42 (0.11-1.53)</td>
<td>0.25 (0.07-0.94)</td>
</tr>
<tr>
<td>20-25</td>
<td>1.00 (reference)</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>25-30</td>
<td>0.66 (0.36-1.02)</td>
<td>1.45 (0.87-2.42)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>0.21 (0.07-0.62)</td>
<td>1.12 (0.52-2.41)</td>
</tr>
<tr>
<td>Iron supplements</td>
<td>-</td>
<td>2.16 (1.10-4.24)</td>
</tr>
<tr>
<td>Alcohol &gt; 2 units/day</td>
<td>0.58 (0.32-1.05)</td>
<td>-</td>
</tr>
</tbody>
</table>

TS, transferrin saturation; SF, serum ferritin; adj, adjusted for the other variables in the model; familial iron severity, the value of TS of the proband in case elevated TS was studied and the value of SF of the proband divided by the reference value in case of elevated SF. Selection was performed when genotype, age, gender and severity were already included; * C282Y/WT includes C282Y/WT, C282Y/unknown; ** WT/WT includes H63D/H63D, H63D/WT, WT/WT; -, not selected.

TABLE 4 shows the adjusted odd ratios of the family-degree model of elevated TS and of elevated SF. The variables to predict elevated TS in this family-degree model only reached the level of borderline significance resulting in a low fit and a moderate discriminatory power (R-square = 4.0%, AUC = 61.7%). The factors predicting an elevated risk for increased SF were comparable to the factors mentioned in TABLE 3, though their influence appeared to be less clearly; familial iron severity.
Determinants for the phenotypic expression of iron overload in the HEFAS population

(OR 1.04, 95%CI 1.01-1.08) and age at testing (OR 1.02, 95%CI 1.003-1.05). The percentage explained variance and the discriminatory power were only slightly better then in the family-degree model of elevated TS (R-square= 15.3%, AUC=70.3%).

In conclusion, we found that the genotype models outperform by far the family-degree models using both the percentage explained variance and the discriminatory power.

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>The adjusted odds ratios of the family-degree model of elevated TS and SF values, among first-degree family members, using multivariate logistic regression with selection procedure.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Elevated TS</td>
</tr>
<tr>
<td></td>
<td>( OR_{adj} ) (95%CI)</td>
</tr>
<tr>
<td>Family degree</td>
<td></td>
</tr>
<tr>
<td>siblings</td>
<td>1.82 (0.67-4.98)</td>
</tr>
<tr>
<td>children</td>
<td>1.80 (0.51-6.29)</td>
</tr>
<tr>
<td>parents</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>1.51 (0.98-2.33)</td>
</tr>
<tr>
<td>female</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Familial iron severity</td>
<td></td>
</tr>
<tr>
<td>1.00 (0.99-1.01)</td>
<td>1.04 (1.01-1.08)</td>
</tr>
<tr>
<td>Age at testing</td>
<td>1.01 (0.99-1.03)</td>
</tr>
<tr>
<td>Meat consumption</td>
<td>-</td>
</tr>
<tr>
<td>Alcohol &gt; 2 units/day</td>
<td>0.65 (0.41-1.04)</td>
</tr>
</tbody>
</table>

TS, transferrin saturation; SF, serum ferritin; adj, adjusted for the other variables in the model; familial iron severity, the value of TS of the proband in case elevated TS was studied and the value of SF of the proband divided by the reference value in case of elevated SF. Selection was performed when genotype, age, gender and severity were already included; -, not selected.

FIGURE 1 shows the observed elevated SF and estimated probability of elevated SF, using the final genotype model. This figure shows that black spots in figure A well agree with the larger bubbles in figure B, indicating the large discriminatory power of our model. The black spots in between the open spots in figure A may indicate that more variables than age and familial severity alone determine (completely) the elevated SF level. Small bubbles in between large bubbles in figure B indicate the effect of other variables than age and familial severity used in the model to predict elevated SF.
Chapter 7

C282Y homozygous

Observed (A)  Expected (B)

C282Y/ H63D, compound heterozygous

Observed (A)  Expected (B)

**Figure 1** The observed (A) and predicted probability (B) of elevated serum ferritin concentration (SF) by severity of iron overload in the family and by age of first-degree family members with homozygous genotype or with compound heterozygous genotype. The black spots in panel A indicate family members with elevated SF and the size of the bubbles in panel B indicate the predicted probability, using the final multivariate logistic regression model. Severity of iron overload in the family was defined as the value of SF of the proband divided by the reference value of the local laboratory.

**Penetrance of Iron Overload**

Finally the risk for a C282Y homozygous FDFM to develop iron overload is presented in Table 5 (males) and Table 6 (females) by age, BMI, use of iron supplements and familial iron severity. For
instance a 50 yr old male, with a BMI of 25 kg/m², not using iron supplements, with a related proband presenting with an SF level of 4 times the reference value, has an estimated risk for iron overload of 0.80 (95%CI 0.69-0.87).

DISCUSSION

The potentially positive effects of family screening for HH to prevent iron accumulation and its related morbidity and mortality in individuals at risk for HH, calls for a thorough investigation of the usefulness of family screening for the early detection of HH, in the light of the discussion on the penetrance of the HFE-gene mutation [22-25]. Recently, we demonstrated an increased HH related morbidity in family members of probands with clinically detected HH compared to an age and gender matched healthy population (chapter 6 of this thesis). In the present study we investigated the HFE-gene mutation penetrance in the FDFM of these families by searching for co-factors determining the amount of accumulated iron. Studying the FDFM of clinically detected HH patients limits ascertainment bias and increases the chance on finding genetic and environmental factors that will predict iron accumulation. As expected, we showed that the risk for iron overload is strongly related to the genotype of the FDFM, e.g. C282Y homozygosity and compound heterozygosity. Next to this, an older age at testing for iron accumulation, a more severe iron overload in the related proband at presentation and the use of iron supplements all contributed significantly to the prediction of iron overload in the FDFM. In contrast, a low BMI protected against iron accumulation, whereas the family degree itself (sibling, parents, child) provided no additional information.

In our population HFE-genotype was the strongest predictor for the accumulation of iron, a finding ascertained by others previously [7, 26-28]. However, we are the first to confirm a positive relationship between the severity of iron overload found in a proband and that detected in the related FDFM, indicating the existence of HFE-genotype penetrance modifying factors, such as other co-inherited genes or environmental family related factors. Previously, Whiting et al already observed a concordance of iron indices in homozygote and heterozygote sibling pairs in hemochromatosis families, suggesting the existence of these familial modifiable factors [29]. Mura et al, however, could not demonstrate a familial predisposition for iron overload, as they found no correlation between TS or SF between sex matched homozygous sibling pairs of which one was clinically diagnosed as HH and one exhibited only total body iron overload [30]. McCune et al also looked at a familial predisposition of HFE-gene expression. They compared the amount of iron overload of C282Y homozygous relatives of clinically affected C282Y homozygous index cases with the amount of iron
<table>
<thead>
<tr>
<th>age (yr)*</th>
<th><strong>BMI (kg/m²)</strong></th>
<th><strong>use of iron supplements</strong></th>
<th><strong>familial iron severity</strong></th>
<th><strong>no use of iron supplements</strong></th>
<th><strong>familial iron severity</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>yes</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>0.45 (0.15-0.78)</td>
<td>0.46 (0.16-0.79)</td>
<td>0.48 (0.17-0.80)</td>
<td>0.49 (0.18-0.81)</td>
<td>0.50 (0.19-0.82)</td>
</tr>
<tr>
<td></td>
<td>0.79 (0.60-0.90)</td>
<td>0.80 (0.61-0.91)</td>
<td>0.81 (0.62-0.91)</td>
<td>0.82 (0.64-0.92)</td>
<td>0.83 (0.65-0.92)</td>
</tr>
<tr>
<td></td>
<td>0.78 (0.53-0.92)</td>
<td>0.79 (0.55-0.92)</td>
<td>0.80 (0.56-0.92)</td>
<td>0.81 (0.58-0.93)</td>
<td>0.82 (0.64-0.92)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>0.52 (0.20-0.83)</td>
<td>0.54 (0.21-0.84)</td>
<td>0.55 (0.22-0.84)</td>
<td>0.57 (0.23-0.85)</td>
<td>0.58 (0.24-0.86)</td>
</tr>
<tr>
<td></td>
<td>0.84 (0.67-0.93)</td>
<td>0.84 (0.69-0.93)</td>
<td>0.85 (0.71-0.93)</td>
<td>0.86 (0.71-0.94)</td>
<td>0.87 (0.72-0.94)</td>
</tr>
<tr>
<td></td>
<td>0.83 (0.62-0.93)</td>
<td>0.84 (0.63-0.94)</td>
<td>0.84 (0.64-0.94)</td>
<td>0.85 (0.66-0.94)</td>
<td>0.86 (0.67-0.94)</td>
</tr>
<tr>
<td>&gt;30</td>
<td>0.60 (0.25-0.87)</td>
<td>0.61 (0.27-0.87)</td>
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* age at time the SF was measured. ** familial iron severity, the value of SF of the proband divided by the reference value.
<table>
<thead>
<tr>
<th>Age (yr)*</th>
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<th>Use of Iron Supplements</th>
<th>Familial Iron Severity**</th>
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<td>0.87 (0.72-0.95)</td>
<td>0.88 (0.73-0.95)</td>
</tr>
</tbody>
</table>

* age at time the SF was measured; ** familial iron severity, the value of SF of the proband divided by the reference value.
overload found in C282Y homozygous relatives of C282Y homozygous blood donors detected by genetic screening [31]. After correcting for potential iron accumulation modifying parameters, the multivariate analyses showed that, in contrast to our findings, being familial related to the clinically affected proband group was no longer a significant risk factor for iron overload. This could be explained by the difference in definition of iron overload in the proband, as we defined the severity of iron overload in the proband by the SF level in contrast to McCune, who defined it by the existence of clinical symptoms in the proband [32].

Surprisingly, our study revealed that the consumption of alcohol was not a predictor for iron accumulation in contrast to what others found [32]. This is most likely due to the fact that we chose a low alcohol intake cut-off value to discriminate between low and high alcohol intake, diminishing the effect of the severe drinkers. The finding that gender did not add any predictive value for iron overload in contrast to findings in literature, is explained by the fact that we used normal SF values already corrected for gender and pre- and post menopausal period. This study also revealed that further standardisation of the SF measurements is requested to make results between laboratories better interpretable. The potentially protective effect for iron overload by a low BMI (<20 kg/m^2) is presumably due to the increased number of individuals in this group with severe diseases inducing iron storage depletion.

Interesting for screening HH patients is the observation that individuals with a high BMI (>30 kg/m^2) had a lower TS. Recently, Laine et al already claimed that the phenotypic expression of C282Y homozygous women, measured by TS, depended on their BMI, as 82% of the women with a BMI >27 kg/m^2 were non-expressing hemochromatosis patients [33]. Bekri et al gave an explanation as they found that HAMP (hepcidin) mRNA expression was increased in adipose tissue of obese patients [34]. As hepcidin decreases the intestinal iron uptake and the reticular endothelial system iron release, these increased hepcidin levels are predicted to result in lowered TS levels. Indeed, they found low TS levels in 68% of the obese patients and 24% of them even presented with anaemia. For HH patients one could postulate that the increased hepcidin expression in the adipose tissue compensates for the diminished hepcidin expression in the liver, protecting these severe obese patients from iron overload. It is, however, not clear whether the SF in these patients is an accurate reflection of the actual iron accumulation found by liver biopsy. Indeed, even more complicating is the finding that hepatic fat content, visceral fat area and subcutaneous fat are positively correlated with SF levels, as with metabolic syndrome and insulin resistance in non-HFE related populations [35-37]. This even suggests a (false positive) increase in SF, in contrast to the decrease found for TS in these obese individuals. Taken together, the relevance of a high BMI for screening for HH remains unclear. The confirmation of the found decreases in TS levels or elevations in SF levels with
histological proven iron overload by liver biopsy, in a HFE related population with a BMI>30 kg/m², is recommended.

The strong relation between HFE-genotype and iron overload we found in the HEFAS families underlined the importance of family screening. However, for clinical relevance we still have to prove the positive relationship between iron accumulation and the development of iron overload related disease and organ failure in our population. Though, the existence of the relation is very plausible, as Powell showed recently. He investigated 672 essential asymptomatic C282Y homozygous individuals identified by family screening or health checks and showed that hepatic fibrosis and cirrhosis correlated significantly with the hepatic iron concentrations [38]. Even more, a significant hepatic fibrosis was frequently found in asymptomatic subjects with iron overload, which was reversible by phlebotomies when no cirrhosis was present urging again early screening on iron overload even if no complaints yet existed.

In the present study we showed that HFE-genotype, age at testing for HH, severity of iron overload within the family, BMI and the intake of iron supplements are the most important predictive factors for developing iron overload in FDFM of clinically diagnosed C282Y homozygous probands. Incorporating these factors in the screening procedures of FDFM for HH should be helpful to identify those individuals most at risk for iron overload and subsequently reduces unnecessary follow-up and treatment of those who will never develop any iron accumulation.

ACKNOWLEDGEMENTS

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(1) Bacon BR. Hemochromatosis: Diagnosis and management. Gastroenterology 2001;120:718-25.


Determinants for the phenotypic expression of iron overload in the HEFAS population


Determinants for hemochromatosis related diseases in Dutch families with HFE-related hemochromatosis. The HEmochromatosis FAmily Study (HEFAS)

Submitted

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ABSTRACT

The clinical penetrance of HFE-related hereditary hemochromatosis (HH) is highly variable and may be influenced by multiple genes, lifestyle and environmental factors, complicating early screening options to prevent iron overload related disease.

The aim of this study was to assess the presence of HH related disease in C282Y homozygous siblings of C282Y homozygous probands with clinically detected HFE-related HH and identify factors predictive for the iron overload related disease within these siblings. To this end C282Y homozygous and non-homozygous siblings were compared regarding serum iron parameters and using structured questionnaires covering general health, lifestyle factors and HH related disease.

In total 428 siblings participated in the study of whom 110 were C282Y homozygous, compromising 53 men (48%). Compared to non-homozygous C282Y siblings, C282Y homozygous siblings mentioned to have more often joint pain (odds ratio (OR) 1.88, 95% confidence interval (95%CI) 1.19-2.99), liver disease (OR 2.90, 95%CI 1.27-6.62) and rheumatism (OR 2.76, 95%CI 1.71-4.46) Using multivariate logistic regression modelling genotype (OR 2.29, 95%CI 1.04-5.02), age (OR 1.07, 95%CI 1.04-1.09) and gender (OR 1.71, 95%CI 1.04-2.80) were predictive for the development of iron overload related disease. With genotype in the model, neither the serum iron parameters, nor lifestyle factors as BMI, smoking or alcohol use added to the prediction of iron overload related disease.

We report that HFE-related disease is increased in the C282Y homozygous siblings stressing the importance of family screening. Knowledge on genotype, gender and age, but not of lifestyle factors, appear to be instrumental in the design of a family screening program that is most cost-effective.

INTRODUCTION

Hereditary hemochromatosis (HH) is an autosomal recessive disease characterized by excessive iron absorption in the intestines leading to iron storage in parenchymal organs. Clinical features of adult onset HFE-related HH are vague and generally consist of complaints such as fatigue, weakness, arthopathy and abdominal pain. Diabetes mellitus, hepatomegaly, abnormal liver tests, impotence, hypothyroidism, cardiomyopathy and hepatocellular carcinoma are described as some of the more advanced problems related to the progressive iron deposition in the internal organs, although the relation with iron overload is not thoroughly proven for all of them [1-7]. As genetic screening became possible, HFE-related HH was regarded as one of the most common genetic disorders of northern Europeans, with approximately five per 1000 individuals being C282Y homozygous [8]. It appeared that early diagnosis and therapeutic phlebotomy effectively prevented the development of tissue
damage and resulted in long term survival similar to the general population [9-11]. Regarding the high prevalence of homozygous individuals and the benefits of an early treatment, population based screening seemed very compelling.

However, a considerable variability in symptomatology and severity of the HFE-related iron overload disease exists, even among patients with identical mutations. This is indicative for powerful effects of other modifying genes, specific environmental influences, other diseases or lifestyle [3, 6, 7]. Predictive DNA testing in asymptomatic individuals seems therefore insufficient to estimate the future risk of disease. This is expected to be different in families in which HFE-related iron overload exists. Family members that share the activity of other modifiable genes, environmental influences and lifestyle with the symptomatic proband, share probably also the clinical penetrance of the HFE-gene mutation for iron overload, making family screening promising and cost-effective, especially if contributing factors are elucidated. However, until now no clearly defined laboratory or patient characteristics are available to risk-stratify patients into groups that are more or less likely to develop overt disease. Even more, as scored by illness and not iron overload alone, equal clinical penetrance of HFE-related disease is still not adequately confirmed in the family members of the clinically diagnosed probands [12].

Therefore, the current study aims to provide evidence for the existence of increased illness in C282Y homozygous siblings of clinically diagnosed C282Y homozygous probands, compared to their non-homozygous siblings. Its second objective is to explore factors determining the clinical expression of the HFE-related disease in these C282Y homozygous siblings. These outcomes will be helpful to define among the siblings a high-risk group for iron overload related disease, potentially increasing the effectiveness of family screening.

METHODS

STUDIED POPULATION
The 428 C282Y homozygous siblings studied all participated in the HEmochromatosis FAmily Study (HEFAS), which encompasses 224 C282Y homozygous probands with clinically detected HH and 735 first-degree family members, all at least 18 years of age, as describe elsewhere (chapter 6 of this thesis).

INCLUSION CRITERIA
Only subjects who gave written informed consent were included in the study. Probands were formerly diagnosed by their clinical signs and symptoms related to iron overload and C282Y-homozygosity. Iron
accumulation was confirmed by initial transferrin saturation (TS) and serum ferritin (SF) values exceeding the reference value thresholds; TS > 50% for both men and women, SF ≥ 280 µg/L for men, SF > 80 µg/L for women under the age of 50, and SF ≥ 180 µg/L for women aged ≥ 50 years, or corresponding values for SF depending on the reference values of the laboratories. When either one or both pre-treatment serum iron parameters were not available, the presence of iron overload was alternatively confirmed by liver biopsy (grade 3 iron deposition according to Sindram) performed before treatment was started or by the number of phlebotomies required to normalize SF (males ≥ 22 phlebotomies, 5 g chelatable iron; females ≥ 13 phlebotomies, 3 g chelatable iron) [2, 13].

**QUESTIONNAIRES**

All participants were asked to fill out a questionnaire comprising a large number of questions on demographics, lifestyle (e.g. smoking, use of alcohol, diet), health status (e.g. exercise and fatigue (scored by shortened fatigue questionnaire score [14]), general medical history (surgery, diseases diagnosed or treated), HH related medical history, and family structure.

**LABORATORY DATA**

Data on the included siblings were extracted from medical records of the participating hospitals or acquired from the physicians involved in diagnosis and treatment of the patients. Information on iron parameters (TS and SF) and liver biopsy of the participants was obtained at the time of diagnosis or screening of HH, whereas data on HFE-genotype and especially on the number of phlebotomies were also collected at points in time after the initial investigations. Whenever the siblings declared to never have been tested for HH, counselling and blood testing by their general practitioner was offered. The TS and SF were quantified using validated, standardized, routine laboratory methods. HFE-genetic test results were obtained from routinely used genetic tests.

**STATISTICAL METHODS**

In this study we aimed at distinguishing the siblings, of probands with clinically detected HFE-related HH, at risk for iron accumulation related disease from those not at risk. Univariate logistic regression technique was used to study the differences in HH related illness and iron parameters between C282Y homozygous siblings vs. non-homozygous siblings. For this purpose, elevated TS was defined as TS above 50%, elevated SF as SF above the gender-and calendar time-specific local laboratory reference values. In case no SF reference value was given, the 67-percentile of all reference values was used. Different reference values for premenopausal and postmenopausal women were taken into account when provided by the laboratories. Furthermore, ‘Hemochromatosis related disease’ was defined as presence of diabetes mellitus, liver disease, rheumatism, fatigue and
cardiovascular disease. The disease prevalences (n) and the crude odds ratios (ORs) with 95% confidence intervals (CIs) are presented.

Multivariate logistic regression with stepwise selection procedures was used to identify variables that, in addition to genotype, contributed independently to the risk of 'Hemochromatosis related disease'. Putative disease determining variables used in the selection procedure were genotype, iron parameters, age at participation, BMI, smoking and alcohol use. The adjusted ORs with 95% CIs of the final model are presented. The R-square is presented to indicate the total percentage explained variance in the outcome and the area under the curve (AUC) of the receiver operating characteristic (ROC) curve is presented as measure of predictive discrimination.

All statistical analyses were performed using SAS version 8.2.

RESULTS

DEMOGRAPHICS

In the study participated 428 siblings of whom 110 were C282Y homozygous. Of the homozygous siblings 48.2% (53) were male, compared to 45.6% (145) of the non-homozygous siblings. The age at participation was similar for both groups. More homozygous siblings ever smoked, whereas the alcohol consumption in both groups was similar (TABLE 1). The median (range) values of TS of the homozygous siblings amounted 78.8% (15.8-108.6%) against 31.5% (2.2-103.9%) for the non-homozygous subjects. For the SF these values were 585 µg/L (14-1153 µg/L) against 110 µg/L (SD 3-196 µg/L).

DISEASE PREVALENCE

TABLE 2 shows the comparison of disease prevalence of the homozygous and of the non-homozygous sibling population, together with the ORs. Homozygous siblings show a significantly higher risk for iron overload than the non-homozygous siblings, with for TS>50% a high OR of 39.08 (95%CI 20.03-76.24) and for SF above normal OR 17.31 (95%CI 9.79-30.61). Furthermore, the homozygous siblings are distinguished by a significant elevated risk for joint pain (OR 1.88, 95%CI 1.19-2.99), liver disease (OR 2.90, 95% CI 1.27-6.62) and rheumatism (OR 2.76, 95%CI 1.71-4.46) compared to the non-homozygous siblings. Even so, there is a significant difference in the hemochromatosis related diseases between the C282Y homozygous siblings and the other genotypes.
TABLE 1  
Demographics of the siblings of the HEFAS population

<table>
<thead>
<tr>
<th></th>
<th>C282Y homozygous genotype</th>
<th>non-homozygous genotype*</th>
<th>p-value</th>
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<td>n^g (%) / median (SD)</td>
<td>n^g (%) / median (SD)</td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>53 (48.2)</td>
<td>145 (45.6)</td>
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<tr>
<td>Age (at participation, yr)</td>
<td>54.0 (9.8)</td>
<td>54.0 (11.3)</td>
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<tr>
<td>Body Mass Index (kg/m^2)</td>
<td>25.4 (3.5)</td>
<td>25.9 (4.4)</td>
<td>0.24</td>
</tr>
<tr>
<td>Smoking (ever)</td>
<td>86 (78.9)</td>
<td>203 (65.1)</td>
<td>0.008</td>
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<tr>
<td>Alcohol &gt; 2 units/day</td>
<td>89 (81.7)</td>
<td>261 (83.7)</td>
<td>0.66</td>
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* contains C282Y/H63D, C282Y/WT, C282Y/unknown, H63D/H63D, H63D/WT, WT/WT; n^g, number of siblings; SD, standard deviation.

Of those factors that were thought to influence the disease penetrance in the sibling group, multivariate analyses show that C282Y homozygous genotype (OR 2.29, 95%CI 1.04-5.02), age (OR 1.07, 95%CI 1.04-1.09) and gender (OR 1.71, 95%CI 1.04-2.80) are correlated with the development of iron overload related disease (TABLE 3). The iron parameters have no significant input here, however when the predictive value of the iron parameters is analyzed in a group of siblings above the age of 55 yrs, the input of the SF value is also significant, with a decreased influence of gender (data not shown). BMI, smoking and alcohol use have no additional predictive value on the development of morbidity. In total 20% (R-square) of the variance in this model could be explained by the selected variables and the discriminatory power is 73% (AUC).

DISCUSSION

Family screening seems very compelling for the early detection of HFE-related HH as the penetrance of the HFE-gene mutations is thought to be higher in families with HFE-related HH compared to the normal population, due to the sharing of activity of other iron accumulation modifiable genes, environmental influences and lifestyle with the symptomatic proband. In former studies we have already demonstrated the existence of a higher morbidity in first-degree family members of probands with clinically detected HH and the existence of iron overload in these first-degree family members related to genotype, severity of iron overload in the proband diagnosed, the age of testing and the BMI (chapters 6 and 7 of this thesis). In line with these outcomes this study reveals that the clinical
Determinants for the hemochromatosis related disease in the HEFAS population

### Table 2: Disease prevalence and crude odds ratios of disease outcomes for homozygous siblings vs. other genotype siblings using logistic regression

<table>
<thead>
<tr>
<th>Iron parameters</th>
<th>Disease prevalence</th>
<th>Crude OR homozygous vs. non homozygous* OR (95%CI)</th>
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<tbody>
<tr>
<td>TS &gt; 50%</td>
<td>84 / 35</td>
<td>39.08 (20.03-76.24)</td>
</tr>
<tr>
<td>SF above normal (µmol/L)</td>
<td>84 / 49</td>
<td>17.31 (9.79-30.61)</td>
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</table>

<table>
<thead>
<tr>
<th>Morbidity</th>
<th>Disease prevalence</th>
<th>Crude OR homozygous vs. non homozygous* OR (95%CI)</th>
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<tbody>
<tr>
<td>anemia</td>
<td>12 / 43</td>
<td>0.80 (0.40-1.60)</td>
</tr>
<tr>
<td>cancer</td>
<td>6 / 15</td>
<td>1.20 (0.45-3.19)</td>
</tr>
<tr>
<td>cardiovascular disease</td>
<td>12 / 33</td>
<td>1.11 (0.53-2.19)</td>
</tr>
<tr>
<td>diabetes mellitus</td>
<td>7 / 13</td>
<td>1.66 (0.64-4.29)</td>
</tr>
<tr>
<td>fatigue**</td>
<td>18 / 38</td>
<td>1.44 (0.78-2.66)</td>
</tr>
<tr>
<td>fertility disorders</td>
<td>5 / 7</td>
<td>2.13 (0.66-6.91)</td>
</tr>
<tr>
<td>hypercholesterolemia</td>
<td>17 / 57</td>
<td>0.88 (0.48-1.61)</td>
</tr>
<tr>
<td>hypertension</td>
<td>40 / 92</td>
<td>1.44 (0.90-2.33)</td>
</tr>
<tr>
<td>hypothyreoida</td>
<td>3 / 12</td>
<td>0.69 (0.19-2.48)</td>
</tr>
<tr>
<td>impotence (male only)</td>
<td>13 / 23</td>
<td>1.62 (0.75-3.52)</td>
</tr>
<tr>
<td>joint pain</td>
<td>67 / 150</td>
<td>1.88 (1.19–2.99)</td>
</tr>
<tr>
<td>liver disease</td>
<td>12 / 13</td>
<td>2.90 (1.27-6.62)</td>
</tr>
<tr>
<td>osteoporosis</td>
<td>9 / 23</td>
<td>1.21 (0.54-2.74)</td>
</tr>
<tr>
<td>porphyria cutanea tarda</td>
<td>2 / 4</td>
<td>0.63 (0.11-3.49)</td>
</tr>
<tr>
<td>rheumatism</td>
<td>25 / 84</td>
<td>2.76 (1.71-4.46)</td>
</tr>
<tr>
<td>Hemochromatosis related disease*</td>
<td>71 / 131</td>
<td>2.82 (1.73-4.61)</td>
</tr>
</tbody>
</table>

n², number of siblings; OR, Odds Ratio; CI, confidence interval; TS, transferrin saturation; SF, serum ferritin; * contains C282Y/H63D, C282Y/WT, C282Y/unknown, H63D/H63D, H63D/WT, WT/WT; ** fatigue scored using the shortened fatigue questionnaire score [14]; † hemochromatosis related diseases: diabetes mellitus, liver disease, rheumatism, fatigue (score \( \geq \) 18) and cardiovascular disease.
expression of symptoms described to HFE-related HH are indeed with high morbidity related to C282Y homozygosity, detecting more HH related illness in homozygous siblings than in siblings with other genotypes. Clinical penetrance determining factors are gender and age of the siblings.

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>The adjusted odds ratios of disease for homozygous siblings vs non-homozygous siblings, using multivariate logistic regression with forward selection procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>OR (95%CI)</td>
</tr>
<tr>
<td>C282Y/C282Y</td>
<td>2.29 (1.04-5.02)</td>
</tr>
<tr>
<td>C282Y/H63D</td>
<td>0.68 (0.22-2.11)</td>
</tr>
<tr>
<td>Other genotype</td>
<td>1.00 (reference)</td>
</tr>
<tr>
<td>Iron parameters</td>
<td></td>
</tr>
<tr>
<td>TS &gt;50%</td>
<td>0.90 (0.43-1.90)</td>
</tr>
<tr>
<td>SF above normal (µmol/L)</td>
<td>1.53 (0.79-2.98)</td>
</tr>
<tr>
<td>Age (at participation, yr)</td>
<td>1.07 (1.04-1.09)</td>
</tr>
<tr>
<td>Gender</td>
<td>1.71 (1.04-2.80)</td>
</tr>
</tbody>
</table>

OR, Odds Ratio; CI, confidence interval; * includes C282Y/H63D, C282Y/WT, C282Y/unknown, H63D/H63D, H63D/WT and WT/WT; TS, transferrin saturation; SF, serum ferritin; R-square is 20%, as measure of the total percentage explained variance in the outcome; AUC (area under the curve) is 73%, as measure of predictive discrimination. For comparison, an AUC equal to 50% is equivalent to random guessing.

Previously, population screening for C282Y homozygosity seemed very compelling, encouraged by the frequent findings of the homozygous HFE-genotype among overt HH patients. However, large scale population studies as conducted by e.g. Beutler and Olynyk, revealed only a low penetrance of clinical symptoms attributed to the HFE-gene mutations, doubting the adequacy of population screening [15, 16]. As indicated, family members of overt HH patients may form a high-risk group, as they are likely to possess the same additional genetic and environmental factors, which might be needed for clinical manifestation. One major problem studying the clinical penetrance though is that at this moment there is no uniform consensus on the complete clinical disease expression in HH. Of the family studies performed so far, the majority of the outcomes used the biochemical penetrance, i.e. iron accumulation, as end point for evaluation, as this is an earlier and more objective symptom of HH than the clinical symptoms themselves [17-22]. None of the studies, however, provided
Determinants for the hemochromatosis related disease in the HEFAS population

Evidence for a relation between iron overload and increased clinical illness in these families except for the development of liver cirrhosis [12]. The HEFAS study was designed to focus on first-degree relatives of patients with homozygous C282Y related hemochromatosis and to analyse the biochemical as much as the clinical penetrance. To our knowledge we are the first to publish a family study with proband related first-degree family members that shows differences in clinical penetrance scored by disease prevalence between C282Y homozygous and C282Y non-homozygous siblings. We found an increased disease occurrence for joint pain, liver disease and rheumatism.

To optimize screening effectiveness, it may be worthwhile to also include other factors that predict the development of HFE-related morbidity. These yet unknown factors may be identified through large screening studies in which homozygous siblings of C282Y homozygous probands clinically affected are compared with those of clinically unaffected probands. If factors that putatively influence disease penetrance can be correlated to HFE related morbidity, these may be included in screening programs to identify as many individuals at risk with the least effort. Indeed Bulaj et al previously compared clinically unselected homozygous family members of clinically affected C282Y homozygous probands, with clinically unselected homozygous family members of C282Y homozygous probands identified as a result of elevated TS values. Disease related conditions were defined as liver fibrosis or cirrhosis, elevated liver enzymes of no other known cause than iron overload or radiographically confirmed hemochromatotic arthropathy of the metacarpal-phalangeal joints. They found a slightly higher percentage of subjects with clinically complaints among the relatives related to the clinically affected probands, indicating that it could be worthwhile to further investigate the reason of this difference [23]. In contrast McCune et al found only a difference in hypertension comparing the illness of homozygous siblings of clinically detected C282Y homozygous probands with those of non-clinically detected C282Y homozygous probands [24]. Even more, when illness was stratified for iron phenotype, only a significant difference for aspartate aminotransferase elevation was found. However, with another study design we were able to point out that there is, indeed, an elevated disease prevalence related with C282Y homozygosity, correlating with genotype, age and gender.

TS and SF values seem to have no additional predictive value for the development of HH related disease in the genotype model. The genotype is most probably such a strong predictor for an elevated TS, that the predictive value of TS itself disappears. The factor age probably diminishes the additive effect of SF even further. When the predictive value of SF is measured in a population of siblings at older age, e.g. above 55 years, SF significantly attributes as predictor for the development of HH related disease. As McCune et al, we were unable to identify additive value of life style factors or unknown genetic factors on the development of disease. However, in our multivariate analyses the
R-square remained low indicating that still other, not measured, co-expressing genes or environmental influences may influence the disease penetrance.

The consumption of alcohol was not correlated with iron accumulation related disease in contrast to the findings from others [24]. This is most likely due to the fact that we chose a low alcohol intake cut-off value to discriminate between low and high alcohol intake, diminishing the effect of the severe drinkers.

The unique character of the HEFAS consisted of the rigorously completing of biochemical and clinical data of siblings of clinically overt HH patients and enables a direct comparison of HFE-related disease among homozygous and non-homozygous siblings and the determinants influencing the HFE-gene penetrance. This study demonstrates that the presence of diseases and symptoms classically related to hemochromatosis, such as arthralgia, rheumatism, and liver disorders, are significantly more manifested in the homozygous group of siblings. The penetrance of disease is particularly influenced by genotype, age and gender, and at older age by SF.

Taken together homozygous siblings of probands with clinically detected HFE-related HH can be considered a high-risk group for the development of clinically overt HH. They should therefore be screened to detect preclinical cases. With proper treatment morbidity and mortality can be substantially reduced. Therefore, an efficient and cost-effective screening strategy for first-degree relatives of HH patients should be established.

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CHAPTER 9

Viewpoint at the realization of early management of hereditary hemochromatosis
The high HFE-gene mutation frequency and the prolonged asymptomatic and early symptomatic phase of HFE-related hereditary hemochromatosis (HH), in combination with relatively simple therapy and the gain in life expectancy expected from effective treatment, make this disease an ideal target for preventive screening. Before considering a specific screening procedure, however, a series of questions should be addressed that underline the importance and relevance of the screening. In 1968, Wilson and Jungner, together with the WHO, developed their so-called principles of mass screening [1]. Some of these 10 criteria were revised by Whitby in 1974, particularly on the issue of clinical course or prognosis [1, 2]. Looking critically at those principles of population screening it becomes clear that, in contrast to former opinions, relevance of mass screening for HH may be questioned and other screening methods may prove more efficient.

The most important drawback in respect to population screening is that more and more studies are being published stressing the incomplete clinical penetrance of HH in the population genetically at risk, questioning the relevance of the health problem postulated [3-8]. Second, it is not clear which groups or individuals are more prone to develop iron accumulation than others. It seems that HFE-genotype alone is not sufficient to induce HH related disease, but other parameters such as transferrin saturation, serum ferritin levels, age and modifier genes may play a key role. Third, there is little information about the benefits of early treatment as there are no placebo or usual care controlled studies of phlebotomy treatment in patients with HH (due to any cause), nor studies that would allow a valid comparison of early versus delayed medical intervention with curative intention [9]. Taken into account that it is generally believed that phlebotomy treatment is beneficial and has no clinical significant side effects, a randomized placebo controlled trial is considered non-ethical as effective treatment would be withheld from the placebo group or a usual care group [6, 10-13].

Nowadays, only clinically complaint-driven screening is used to identify HH patients and prevent disease progression. However, as discussed in this thesis, this type of screening turned out to be inadequate, partly because there is not enough knowledge of HH among the physicians involved in diagnosing HH and partly because organ damage, causing complaints, is already irreversible.

In HFE-gene related HH a considerable variation in symptomatology and severity of the disease exists, even among patients with identical mutations. The clinical penetrance of the HFE-gene mutation in first-degree family members, who probably also share the activity of other modifier genes, environmental influences and lifestyle, makes it worthwhile to initiate family screening for HH, particular if other putative mutations and contributing factors can efficiently be screened for in these first-degree family members. This is the very reason why family screening for HH has been studied, in particular in this thesis.
As in population screening, also in family screening, the first important issue was to demonstrate the existence of an important health problem. By comparing morbidity in first-degree family members of clinically diagnosed C282Y homozygous probands with morbidity of an age and gender matched population, it was revealed that indeed important health problems were present in the families of clinically diagnosed C282Y homozygotes. The next step was to assess which factors determined who were at particular risk for developing iron overload. Homozygous C282Y mutation and compound heterozygous HFE-gene mutation appeared to have the highest predictive level for iron overload. Besides these two genetic factors, age at testing of serum ferritin and severity of iron overload in the proband correlated very well with iron accumulation in the first-degree family members, as did supplemental intake of iron. Furthermore, a model was developed to identify those first-degree family members at high risk for developing iron storage disease. Subsequently, to underline the necessity of family screening for prevention of further manifestation of HH related disease, the relation between HFE-gene mutations, iron accumulation and disease had to be confirmed. It appeared that joint pain, liver disease and rheumatism were significantly more prevalent in the C282Y homozygous siblings compared to the non-homozygous siblings. Especially age and gender of the C282Y homozygous siblings were co-predictive factors for disease development. The role of serum ferritin was only significant at older age, i.e. after the age of 55 years. Now with strong evidence of a health problem for which acceptable treatment is available, with a distinguished preclinical course from latent to manifest disease and a suitable screening test for detecting this disease in its early (pre-)symptomatic stage, it is useful to identify key factors to implement this form of family screening successfully.

NEXT ON THE IMPLEMENTATION AGENDA

Based on our growing knowledge of the penetrance of the HFE-gene mutations and its co-influencing factors, first of all a cost-effectiveness analysis of family screening is needed. Secondly, a strategy plan on the organization of the screening facility should be developed and organisational questions be answered. What kind of diagnostic facilities and treatment options should be available for the follow-up of abnormalities revealed by the screenings procedures? What has to be done with findings that are neither clearly normal nor obviously abnormal? These plans should also address questions like: What are the implications in terms of resources (education of the public, availability of staff, operating cost) and in terms of introducing on a large scale a family screening program. Third, the social and ethical questions should be taken into account. Stigmatization and discrimination are unwelcome outcomes of screenings procedures. What are the effects on insurance policies? And
finally, some prefer to start with it, before family screening in the Netherlands is allowed to commence at all, it is necessary to address the list of criteria developed by the Netherlands Health Council Committee; “Screening inheritable and congenital disorders” (Gezondheidsraad Commissie; Screening erfelijke en aangeboren aandoeningen) [14].

For the operational and organizational issues to be addressed much can be learned from the Foundation for Tracing Hereditary Hypercholesterolaemia (Stichting Opsporing Erfelijke Hypercholesteroleamia, StOEH) [15, 16]. They have developed a centrally coordinated program for family screening with input from the physicians (General Practitioners (GPs) and medical specialists) on probands in the Netherlands. As a matter of fact the HEmochromatosis FAmily Study (HEFAS) itself was already partially set up using this format, e.g. approximately 40% of the first-degree family members have been prospectively screened. In analogy with StOEH a database was built with family trees of all included families. Family members with unknown blood results were invited by mail to let their blood samples be tested for HFE-gene mutations and iron parameters. Counselling on HH and blood donation took place by the individuals’ own GPs, who also received the final results of the test including a recommendation on how to treat their patient. This advice varied from no necessity for follow-up, to referring the patient to a specialist either for further investigation or for treatment. First-degree family members were not visited. Those who gave no response on our mail invitation were contacted by phone. This resulted in an 85% response rate of the first-degree family members, 15% of them indicated not to desire any further examination. Altogether, a potentially effective family screening design was conducted.

The StOEH-like approach has another important advantage: Central storage of data and blood samples facilitates intermediate evaluations on the (cost-)effectiveness of the program and future research programs on e.g. HFE-mutation penetrance modifying genes, long term disease development, reversibility of complaints, and on the social or ethical impact of family screening.

Still, before a foundation unit searching for hereditary iron overload disease (Stichting Opsporing Erfelijke IJzerstapelingsziekte, StOEIJ) can be initiated decisions should be made about medical knowledge needed in the foundation (for instance on interpreting the outcome results), the most adequate way of counselling for HH, and the medical teaching in the Netherlands needed on diagnosing and treating HH. For the latter the recently developed consensus guidelines for HH diagnosis and treatment (Richtlijn hereditaire hemochromatose, diagnostiek en behandeling van hereditaire hemochromatose) delivers keynote information, as will the implementation of a predictive model for HH as emerging from our HEFAS analyses. As far as research and practice has taught us up to now, for family screening purposes blood samples of screenees need to be tested only for transferrin saturation, serum ferritin and HFE-gene mutations. This together with gender, age, serum
ferritin of the proband (when diagnosed with clinical disease), BMI and use of iron supplements will provide a good estimation of the chance to develop iron overload and its related disease, in the first-degree family member screened. Whenever this risk is elevated, referral should take place to a physician familiar with HH either to confirm or to exclude the suspicion and diagnose alternative pathology causing the aberrant findings.

FURTHER CONTEXTUAL FACTORS

Little is known about the psychosocial impact of family screening for HH. The genetic cascade screening program for familial hypercholesterolemia turned to be highly acceptable to screenees, although there were some reports on social pressure [17]. For HH screening in the general population it appeared that after screening the quality of life and the psychosocial well-being of individuals with a diagnosis of HH without end organ damage was comparable to their unaffected siblings after screening [18]. Similarly, an Australian study on screening for HFE-gene mutations by cheek-brush, found no increase in general anxiety and no significant mental or physical health changes as a result of testing, regardless of genetic test results [19]. Nevertheless, it would be wise to investigate the social and ethical implications for HH related family screening, for instance in the HEFAS families already screened. Also a kind of monitoring system to detect psychological and/or societal utterances in an early phase is proposed.

To be conclusive, the plea of the Netherlands Board of Health Insurances (College voor Zorgverzekeringen, CVZ) to implement a program for family screening on HH, as posed in March 2003, is underlined by the outcomes of this thesis.
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One of the first clinicians who described the clinical syndrome with portal cirrhosis, diabetes mellitus and bronze skin pigmentation was Trousseau in 1865. In 1889, Von Recklinghausen named the syndrome by its current name: hemochromatosis. Hereafter Sheldon concluded in 1935 that: "The view advanced as the most reasonable explanation of hemochromatosis that it should be classed as an inborn error of metabolism, which has an overwhelming incidence in males, and which at times actually has a familial incidence. It concerns the inner metabolism of probably all the cells of the body." It lasted until 1996 before a genetic basis for the clinical symptoms was established by the discovery of a homozygous C282Y mutation in the HFE-gene by Feder. Quickly afterwards it became clear that the HFE-gene mutation was probably one of the most common mutations in the North European population leading to inherited metabolic abnormalities. However, despite the rapid scientific progress in the last decades, the exact pathological mechanism for the iron overload disorder is still unknown, but more and more evidence points to a pivotal role for hepcidin deregulation as the main cause of the unbalanced iron metabolism. Formerly, the life-expectance of the hereditary hemochromatosis (HH) patients was quantified as poor. The introduction of phlebotomy treatment, to remove the iron overload from the body, attenuated disease symptoms and brought life expectancy back to the level of healthy persons, when treatment was started before irreversible damage had occurred.

The aim of this thesis was to investigate the potential pathophysiological mechanisms and to reveal an optimal early management strategy for HH. In CHAPTER 2 the changing aspects of HFE-gene related HH are discussed. First seen as a highly frequent HFE-gene related autosomal recessive disease, with a presumed high morbidity and mortality rate, it became a disease characterized by a highly frequent potentially pathological HFE-gene mutation, with various penetrance regarding iron overload and an even lesser penetrance regarding organ dysfunction leading to organ failure and death. This changing view is also reflected in the strategy recommended for the early detection of the HFE-gene related HH. Earlier, population screening seemed to be the most accurate way to prevent instances of disease and death. At the start of the thesis however, family screening has been advocated. It offers the best opportunity for finding C282Y homozygous individuals (25% of the siblings of an diagnosed proband). It also increases the chance to discover family members with the same genetic and environmental influences as the proband, which likewise may engrave their phenotypic expression of HH. But, for an optimal screening strategy, profound background information on the relevance of the health problem involved, and on the clinical penetrance of the mutation screened for is desired. Wilson and Jungner, and later Whitby provided us with criteria useful to determine, if any type of screening should be started.
Before pathological non-transferrin-bound iron (NTBI) related mechanisms of iron overload induced diseases were studied, we examined the standardization of the NTBI measurements in **CHAPTER 3**. The presence of NTBI is thought to be a pathological condition that may enable iron to catalyze redox reactions and contribute to iron accumulation in organs. The exact structure of NTBI is, however, mainly unknown. This led to the development of several diverse quantification methods for NTBI that had never been compared before. The in this thesis performed first comparison of the various methods showed considerable variation in their results. There was a wide spread in mean serum NTBI measured by the collaborating laboratories regarding the between sample and within sample variation. The results obtained with methods based on chelators correlated significantly. However, the NTBI values obtained by the various described methods related differently with the levels of serum transferrin. Therefore, before NTBI can be introduced into clinical practice or can be used for screening purposes, more robust quantification methods should be developed and additional information is needed on its structure and relevance.

In **CHAPTER 4** the role of the body iron in atherosclerotic mechanisms was assessed through determination of the relationship between levels of serum iron parameters, including NTBI, and plasma markers of inflammation and oxidized LDL in patients screened for HH. It appeared that the plasma level of soluble intercellular adhesion molecule-1 (sICAM-1) was positively related to ferritin and to NTBI and negatively to total iron-binding capacity (TIBC). Next, statistically significant higher levels of sICAM-1 were found in subjects in the highest quartile of NTBI compared with the lowest quartile of NTBI. The white blood cell count was positively related to ferritin, whereas high sensitivity C-reactive protein, interleukin 6, interleukin 8, oxidized LDL, oxidized LDL/apolipoprotein B and IgG and IgM antibodies to oxidized LDL were not related to any of the markers of iron status. Thus, we concluded that an excess body iron, reflected by elevated serum ferritin and NTBI and decreased TIBC, is associated with increased plasma level of sICAM-1 but not with markers of *in vivo* LDL oxidation. Enhanced recruitment of inflammatory cells to the extravascular compartment by increased endothelial expression of sICAM-1 may contribute to the suggested increased risk on cardiovascular disease at excess body iron.

After the above-mentioned two studies on NTBI measurement methods and the potential pathophysiological mechanism of NTBI, now in this thesis a transition is made to applied medical research, i.e. the early management of HH through early detection. In **CHAPTER 5** the impact of the introduction of a guideline on the targeted detection of HH is discussed. As HH can present itself with a broad spectrum of complaints and organ dysfunction, it is often difficult for a physician to recognize the disease before irreversible damage has occurred. A multidisciplinary guideline was implemented
in an outpatient department (OPD) at a university hospital, containing recommendations to screen for HH when a patient presented with symptoms or signs possibly related to HH. Retrospectively the diagnostic procedures for patients visiting the OPD in a certain period before and after the introduction of the guideline were compared. Data showed that the number of detected HH patients did not significantly increase, at comparable cost per case detected, with the drawback of many false positive HH diagnoses. A better guideline implementation strategy should increase the awareness of clinicians for HH and guarantee the improvement of the physicians’ interpretation of the results obtained.

In CHAPTER 6 the relevance of the health problems in families with HH was studied. Clinically diagnosed probands with HFE-gene related HH and their first-degree family members (derived from the HEmochromatosis FAmily Study, HEFAS) were compared with an age and gender matched normal population (sampled from the Nijmegen Biomedical Study, NBS) regarding their body mass index (BMI), general health, medication intake, morbidity and mortality. It was shown that the hemochromatosis related diseases were significantly more present in the HH families compared to the normal population. This in contrast to the mortality figures, which turned out to be equal in both populations. These findings justified further research of the value of family screening into the early detection of HH. Furthermore they challenged us to search for environmental and genetic factors that increase the risk for iron overload.

Next, determinants of phenotypic expression of iron overload in Dutch families with HFE-gene related HH were studied in CHAPTER 7. The iron parameters of the first-degree family members of the HEFAS study were related to various possible iron accumulation co-determining factors. Multivariate regression analyses revealed that the amount of accumulated iron was strongly determined by C282Y homozygosity, compound heterozygosity, severity of iron accumulation in the proband and age at testing for serum ferritin. Of the environmental factors supplemental iron intake worsened iron accumulation, while a low BMI (<20 kg/m²) showed a protective effect. Furthermore, a model was developed that is able to predict which first-degree family members are at high risk for developing iron overload and should be followed up with proper treatment.

In CHAPTER 8 the penetrance of the disease within the HEFAS C282Y homozygous siblings was quantified. The penetrance was clearly higher in the homozygous siblings than in the non-homozygous siblings for joint pain, liver diseases, rheumatism and hemochromatosis related diseases, defined as diabetes mellitus, liver disease, rheumatism, fatigue and cardiovascular disease. Predictive for the penetrance were genotype, age and gender, whereas serum ferritin levels were
only correlated with disease at older age, i.e. above the age of 55 years. We could not prove the additive value of life-style factors in the development of HH related diseases, although the R-square statistic of only 20% for the total comparison of disease for homozygous siblings vs. non-homozygous siblings, could indicate the existence of not yet identified co-expressing genes or environmental influences.

In CHAPTER 9 a viewpoint is given on the realization of the early management of HH through family screening. Based on the HEFAS outcomes there is indeed a health problem within HFE-gene related families; i.e. there is increased iron overload, already manifestating in increased morbidity. Furthermore, a suitable screening test for detecting the disease is available and effective. One should look now for additional factors that may help to successfully implement family screening. Cost-effectiveness analysis should be initiated and a strategy plan for the organization of a screening facility should be set up. In tandem to these logistic activities ethical and social implications of HH related family screening should be checked against the criteria developed by the Netherlands Health Council Committee “Screening inheritable and congenital disorders”, before finally introducing HH related family screening in the Netherlands.
SAMENVATTING
Een van de eerste medici die het klinische syndroom beschreef van levercirrhose, diabetes mellitus en gebronsde huid was Trousseau in 1865. Von Recklinghausen noemde dit ziektebeeld in 1889 voor het eerste bij zijn huidige naam: hemochromatose. In 1935 schreef Sheldon: “De meeste waarschijnlijke verklaring voor het ontstaan van hemochromatose is een metabole ziekte, met een hoge incidentie bij mannen en een regelmatig familair voorkomen. Het betreft een ziekte die waarschijnlijk op alle lichaamscellen van invloed is.” Het duurde uiteindelijk tot 1996 voordat Feder de genetische basis van de klinische symptomen beschreef met de ontdekking van de homozygote C282Y mutatie in het HFE-gen. Vlot hierna werd duidelijk dat de C282Y-gen mutatie binnen de Noord-Europese populatie waarschijnlijk een van de meest voorkomende mutaties was verantwoordelijk voor een metabole ziekte. Het exacte pathologische mechanisme van het ontstaan van ijzerstapeling is echter, ondanks de snelle wetenschappelijke vooruitgang de afgelopen decennia, nog steeds niet opgehelderd. Er bestaan wel steeds meer aanwijzingen voor een sleutelrol voor het eiwit hepcidine bij het ontstaan van deze ijzerstapelingsziekte. In vroegere tijden was de levensverwachting van patiënten met hereditaire hemochromatose (HH) slecht. Maar met de introductie van aderlatingen om op deze manier ijzer uit het lichaam te onttrekken, namen de ziektesymptomen af en werd de levensverwachting van de HH-patiënten teruggebracht naar het niveau van de gezonde bevolking, mits de behandeling met flebotomieën werd gestart voordat irreversibele lichaamsschade was ontstaan.

Het doel van dit proefschrift was onderzoek te verrichten naar de mogelijke pathofysiologische rol van het niet-transferrine gebonden ijzer (non-transferrin-bound iron = NTBI) en te komen tot een optimale strategie waarmee HH in een vroeg stadium kan worden opgespoord en behandeld. In HOOFDSTUK 2 worden de veranderde inzichten van het HFE-gen gerelateerde ziektebeeld besproken. Hoewel HH eerder gezien werd als een veel voorkomende autosomaal recessieve aandoening met een hoge morbiditeit en hoge mortaliteit, wordt de ziekte tegenwoordig meer gekarakteriseerd door de aanwezigheid van de frequent voorkomende homozygote C282Y-mutatie met een zeer wisselende penetrantie, die uiteindelijk in een gedeeltelijke gemuteerde populatie leidt tot ijzerstapeling, orgaanfalen en overlijden. Deze veranderde kijk op de aandoening komt ook tot uiting in de strategie die wordt voorgesteld om de HFE-gen gerelateerde HH vroegtijdig te ontdekken. Voorheen werd bevolkingsonderzoek als de meest adequate vorm van screening genoemd ter preventie van ziekte en overlijden, maar op dit moment gaat de voorkeur uit naar familie-screening. Deze vorm van screening geeft de meest optimale mogelijkheden om C282Y-homozygoten te vinden, omdat immers 25% van de broers en zussen van een proband homozyoot zal zijn. Daarnaast verhoogt familie-screening de kans op het vinden van familieleden die dezelfde genetische en omgevingsinvloeden hebben ondervonden als de proband, leidend tot de ziekte verschijnselen die bij
HH passen. Echter voor een optimale screeningsstrategie is gedegen achtergrondinformatie nodig over de relevantie van het gezondheidsprobleem in de doelpopulatie en de klinische penetrantie van de ziekte waarop men wil screenen. Eind jaren zestig hebben Wilson en Junger, en begin jaren zeventig ook Whitby, een aantal criteria beschreven die behulpzaam zijn bij de beslissing of, en zo ja, welke vorm van screening geïmplementeerd zou moeten worden.

Voordat we de pathologische mechanismen waarmee NTBI ziekteverschijnselen kan uitlokken hebben bestudeerd, hebben we de standaardisatie van de NTBI-meetmethoden onderzocht en beschreven in HOOFDSTUK 3. De aanwezigheid van NTBI wordt gezien als een potentieel pathologische omstandigheid, waarbij het ijzer in het lichaam gebruikt kan worden om redox-reacties te versnellen, hetgeen kan zorgen voor orgaanschade. De structuur waarin NTBI in het bloed aanwezig is, is onvoldoende bekend. Mede hierdoor zijn er in het verleden diverse meetmethoden ontworpen ter kwantificatie van het NTBI. In dit proefschrift zijn voor het eerst verschillende van deze meetmethoden met elkaar vergeleken, waarbij bleek dat de methoden een aanzienlijke variatie vertoonden in hun uitkomsten. Er was een grote spreiding in de gemiddelde serum NTBI-waarde, zowel voor de waarden van de diverse monsters gemeten door de verschillende laboratoria, als voor de waarden van een specifieke monster gemeten per laboratorium. De resultaten verkregen door middel van chelatiemeetmethoden correleerden significant, maar de diverse NTBI-spiegels gemeten door de verschillende methoden toonden allen een andere relatie tot de gemiddelde serum transferrinewaarde. Concluderend; er dienen beter uitgewerkte kwantificatiemethoden voor het meten van NTBI te worden ontworpen, voordat het gebruik van NTBI kan worden toegepast in de kliniek. Daarnaast dient er meer bekendheid te komen over de preciele samenstelling van het NTBI in het bloed.

In HOOFDSTUK 4 wordt het mechanisme onderzocht waarmee ijzer in het lichaam kan bijdragen aan de ontwikkeling van atherosclerose. Bij patiënten, die voor HH werden gescreend, is gekeken naar de relatie tussen de verschillende hoeveelheden plasma ijzerparameters, waaronder NTBI, en de plasmamarkers voor inflammatie en geoxideerd LDL (low density lipoprotein). Het bleek dat de plasmaspiegel van het oplosbare intracellulaire adhesiemolecuul-1 (soluble intercellular adhesion molecule-1 = sICAM-1) positief is gecorreleerd met ferritine en NTBI en negatief gecorreleerd met de totale ijzerbindingscapaciteit (TIJBC). Daarnaast worden significant hogere hoeveelheden sICAM-1 gevonden bij individuen binnen het hoogst kwartiel van NTBI-spiegels, vergeleken met individuen in het laagste kwartiel van de gemeten NTBI. De hoeveelheid witte bloedcellen is positief gecorreleerd met ferritine, terwijl het hoog sensitieve C-reactieve proteïne (C-reactive protein = CRP) , interleukine 6, interleukine 8, geoxideerd LDL, geoxideerd LDL/apolipoproteïne B en IgG en IgM antilichamen
tegen LDL niet gerelateerd zijn met een van de ijzerparameters. Concluderend; een overmaat aan lichaamsijzer, weergeven door een verhoogd serum ferritine en NTBI en een verlaagde TIBC, is geassocieerd met een verhoogde plasma hoeveelheid van sICAM-1, maar niet met markers van *in vivo* LDL-oxidatie. Mogelijk dat de verhoogde gang van ontstekingscellen naar het extravasculaire compartiment, door de verhoogde endotheliale expressie van sICAM-1, bijdraagt aan het veronderstelde toegenomen risico op cardiovasculaire ziekten bij een overmaat aan lichaamsijzer.

Na de beschrijving van de meetmethoden voor NTBI en het mogelijke pathofysiologische mechanisme waarmee NTBI zijn invloed uitoefent op het cardiovasculair lijden, wordt in het proefschrift een overstap gemaakt naar toegepast medisch-wetenschappelijk onderzoek en wel de aanpak rondom de vroege detectie van HH. In HOOFDSTUK 5 wordt de uitwerking beschreven van de invoering van een protocol voor klinisch geïndiceerd onderzoek voor HH. Omdat HH zich als ziektebeeld kan uiten met een breed spectrum aan klachten en orgaanfalen, is het voor de arts vaak moeilijk om de aandoening te herkennen voordat er irreversibele schade is ontstaan. Daarom werd een multidisciplinair protocol ontworpen met daarin richtlijnen voor screening op HH wanneer zich een patiënt aanbood met klachten of symptomen passende bij HH. Dit protocol werd ingevoerd op de poli van een academisch ziekenhuis. Retrospectief werden de diagnostische procedures die de patiënten hadden ondergaan voor de invoering van het protocol, vergeleken met de procedures die de patiënten ondergingen na de invoering van het protocol. De aantallen gediagnosticeerde patiënten namen, niet significant, toe, tegen een vergelijkbare hoeveelheid gemaakte kosten, met als negatief bijeffect een stijging van de frequentie foutpositieve diagnoses. Een betere implementatie van het protocol zou de bekendheid van de artsen met HH moeten vergroten en de interpretatie van de gevonden resultaten tijdens het screenen van de patiënten op HH verbeteren.

In HOOFDSTUK 6 wordt de relevantie van het met ijzerstapeling gerelateerde gezondheidsprobleem bestudeerd in families met HH. Klinisch gediagnosticeerde probandi met HFE-gen gerelateerde HH en al hun eerstegraads familieleden (allen voortkomend uit de HEmochromatose FAmilie Studie = HEFAS) werden vergeleken met voor leeftijd en geslacht gematchte personen uit de algemene bevolking (genomen uit de Nijmegen Biomedische Studie = NBS). De vergelijking betrof body mass index (BMI), algehele gezondheid, medicatiegebruik, ziekten en doodsoorzaken. Aangetoond kon worden dat de hemochromatose-gerateerde ziekten significant vaker voorkwamen in de HH-families vergeleken met de algehele bevolking. Dit was in tegenstelling tot de mortaliteitscijfers die voor beide populaties vergelijkbaar waren. Deze bevindingen rechtvaardigden verder onderzoek naar de waarde van familiescreening om HH vroeg te detecteren. Daarnaast daagden deze uitkomsten ons uit verder te zoeken naar omgevings- en genetische factoren die een verhoogd risico geven op ijzerstapeling.
Vervolgens worden in HOOFDSTUK 7 factoren beschreven die van invloed zijn op de fenotypische expressie van ijzerstapeling in Nederlandse families met HH. De ijzerparameters van de eerstegraads familieleden uit de HEFAS-studie werden gecorreleerd met verscheidene mogelijke ijzerstapelingsbepalende factoren. Multivariate regressie-analyse liet zien dat de hoeveelheid gestapeld ijzer voornamelijk wordt bepaald door C282Y-homozygotie, compound heterozygotie, ernst van ijzerstapeling bij de probandus en de leeftijd waarop het serum ferritine bepaald werd. Van de omgevingsinvloeden blijkt de inname van ijzertabletten de ijzerstapeling te verergeren, terwijl een lage BMI (<20 kg/m²) een beschermend effect heeft. Daarnaast werd een model ontworpen, waarmee voorspeld kan worden welke eerstegraads familieleden een verhoogd risico hebben op ijzerstapeling en derhalve dienen te worden gevolgd en zonodig behandeld.

In HOOFDSTUK 8 wordt de morbiditeit bij de C282Y-homozygote broers en zussen gekwantificeerd. Er is duidelijk meer morbiditeit bij de homozygote broers en zussen vergeleken met de niet-homozygote broers en zussen qua gewrichtsklachten, leverziekten, reumatische klachten, vermoeidheid en hartvaatziekten. Voorspelling voor een hogere penetratie zijn genotype, leeftijd en geslacht. De serum ferritinespiegels zijn alleen voorspellend bij een oudere leeftijd, zoals boven de 55 jaar. We konden geen leefstijlverschillen aantonen die bijdroegen aan de ontwikkeling van HH-gerelateerde ziekte. De $R^2$ van de totale vergelijking tussen de twee (homozygote en niet-homozygote) groepen bedroeg echter maar 20%, wat kan duiden op de aanwezigheid van nog niet-geïdentificeerde cofactoren.

In HOOFDSTUK 9 wordt een perspectief gegeven voor de realisatie van de vroege diagnostiek van HH. Gebaseerd op de uitkomsten van de HEFAS-studie blijkt er een groot gezondheidsprobleem te bestaan in families met HFE-gen gerelateerde HH; er is een verhoogde ijzerstapeling en een verhoogde ziektelast. Daarnaast bestaat er een adequate en effectieve screeningstest om de ziekte te ontdekken. Derhalve dient er van nu af aan ook gekeken te worden naar factoren die noodzakelijk zijn voor een succesvolle implementatie van familiescreening. Kosteneffectiviteitsanalyses dienen te worden opgezet en er moet een strategisch plan komen voor de organisatie van de familiescreening. Tegelijkertijd met deze logistieke activiteiten dienen de ethische en sociale implicaties van deze familiescreening te worden beschouwd tegen de al ontwikkelde criteria voor screening door de Commissie “Screening erfelijke en aangeboren aandoeningen” van de Gezondheidsraad, voordat uiteindelijk HH-gerelateerde familiescreening in Nederland kan worden geïntroduceerd.
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Jacobs EMG, Verbeek ALM, Kreeftenberg HG, van Deursen CThBM, Marx JJM, Stalenhoef AFH, Swinkels DW, de Vries RA.
Submitted.

FIRST-DEGREE RELATIVES OF C282Y HOMOZYGOUS PROBANDS WITH CLINICALLY DETECTED HEMOCHROMATOSIS HAVE INCREASED MORBIDITY COMPARED TO THE GENERAL POPULATION. THE HEMOCHROMATOSIS FAMILY STUDY (HEFAS).
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De HEFAS studie heeft plaatsgevonden in samenwerking met de onderstaande klinieken

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